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(54) Title: ANTIGEN PROCESSING

(57) Abstract

Genes encoding protein subunits involved in the processing and presentation, in association with MHC class I molecules, of peptide antigens and designated RING4, RING10, RING11 and RING12 are described and claimed together with related proteins and peptides, antibodies, nucleic acids, vectors and other products which are useful in diagnosis and therapy of immune-system disorders and associated diseases.

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ANTIGEN PROCESSING

The present invention relates to various genes and associated products involved in the processing and presentation of antigenic peptides, and to processes for the production and use of such products.

Intracellular protein antigens are believed to be degraded into antigenic peptides lacking any hydrophobic signal sequences, transported to the cell surface and presented, in association with Class I molecules of the Major Histocompatibility Complex (MHC), by various cells of the immune system. However little is known about the mechanism by which such processing, transportation and presentation occurs.

The present inventors have identified a cluster of genes located within the MHC which encode proteins involved in antigen processing and transportation of antigenic peptides. This raises the possibility of a range of diagnostic tests for various dysfunctions of the immune system and for intervention in the antigen presentation process, for instance to enhance the immune response to vaccines and to diminish the immune response associated with autoimmune diseases.

In more detail, the genes involved in the present invention are listed below:

RING 4 encodes a transporter subunit

RING 10 encodes a proteasome subunit

RING 11 encodes a transporter subunit

RING 12 encodes a proteasome subunit

The protein products which are encoded by the genes RING 4 and RING 11 are believed to be subunits of a transporter protein or proteins involved in moving antigenic peptides across the membrane between the cytosol and the endoplasmic reticulum (ER). The protein(s) are believed to consist of a dimer or multimer of similar or identical subunits. Without wishing to be bound by theory it is presently considered most likely that the transporter protein is a heterodimer of one RING 4 subunit and one RING 11 subunit though less likely possibilities include proteins which are homodimers of two RING 4 subunits and proteins which are homodimers of two RING 11 subunits.

The protein products encoded by RINGs 4 and 11 are both members of the ATP binding cassette (ABC) superfamily of transporter proteins. Other members of this superfamily include the human multidrug resistance protein (MDR), the human cystic fibrosis gene product, the white/brown system in drosophila and a series of transporters from bacteria and eukaryotic cells capable of transporting a range of

substrates.

The family of ABC transporters now includes over 30 examples, each of which is specific for a different substrate. Substrates that are transported include sugars, inorganic ions, amino acids, peptides and proteins. The oligopeptide permease in bacteria is known to handle small peptides; the S. cerevisiae STE6 protein transports the α-factor pheromone; in E. Coli the hlyB gene product transports α-haemolysin, a protein of about 107kd, and related transporters export specifically other large polypeptides. In none of these cases does the transported peptide molecule have a typical hydrophobic signal sequence.

The processing of protein antigens is probably undertaken by proteins of the proteasome complex. This is a large intracellular protease (also known as macropain or multicatalytic proteinase complex) composed of at least 13 subunits with broad specificity having the ability to catalyse several proteolytic reactions which is believed to be involved in processing protein antigens. Again, without wishing to be bound by theory, it is presently believed that the RING 10 and RING 12 subunits are involved in formation of the proteasome complex.

The protein product of RING 10 shows a good match with the consensus sequence of active site residues in over 20 members of the subtilisin family of serine proteases.

The present invention will be illustrated with reference

to the figures of the accompanying drawings in which

Fig.1 shows a map of the MHC region indicating the arrangement of the genes RING 4,10,11 and 12 in relation to other known genes of MHC.

Fig.2 gives the complete sequence of RING 4 cDNA

Fig.3 gives the complete sequence of RING 10 cDNA

Fig.4 gives the complete sequence of RING 11 cDNA

Fig.5 gives the complete sequence of RING 12 cDNA

Fig.6 shows a Northern blot of RNA from various cell lines illustrating expression of RING 4.

Fig.7 shows an assignment of potential membrane-spanning regions within the derived amino-acid sequence of RING 4.

Fig.8 shows a comparison of the highly conserved regions of the ATP binding folds of RING 4 and related gene products.

Fig.9 shows a comparison of the hydrophilicity of the RING 4 and RING 11 gene products.

Fig.10 shows a Northern blot of RNA from various lines illustrating expression of RING 11.

Figs.11 & 12 show comparisons of the sequences of RINGs 4 and 11 and other members of the ABC transporter family.

Fig.13 shows the polymorphism of RING 11 in the 3' coding region of the gene.

Fig.14 shows a comparison of the sequences of RING 10

with various proteasome components.

Fig.15 shows diagon plots of cDNA-derived protein sequences related to RING 10.

Fig.16 shows an alignment of a position of RING 10 with various human LMPs.

Fig.17 shows a comparison of RING 10 with various serine protease active sites.

Fig.18 shows Northern blots illustrating expression of RING 10.

Fig.19 shows Northern blots illustrating expression of RING 12.

Fig.20 shows N-terminal amino-acid sequences of RING 12 gene product and related rat and human proteasome subunits.

Fig.21 shows immunoprecipitation of RING 4/11 protein complexes.

Fig.22 shows Southern and Northern blots and the sequence of a mutant RING 11 gene.

Fig.23 and 24 illustrate expression of stable Class I molecules by RING 11A transfectant cells.

Fig.25 illustrates antigen presentation by RING 11A transfectant cells.

Fig.26 shows the directions of transcription of RINGs 4, 10, 11 and 12 and their involvement in antigen processing and presentation.

Fig.27 shows the substitutions in four RING 11 mutants

compared with the RING 11 sequence of Fig.4.

The arrangement of the genes RING 4, 10, 11 and 12 within the MHC is illustrated is Fig.1 and the coding sequences thereof are shown, as cDNA sequences, in Figs. 2 to 5.

In Fig.1 the upper panel shows the main known genes of the Class II region of the short arm of human chromosome 6. The lower panel shows the positions of the RING 4, 9, 10, 11 and 12 genes (RING 9 may not be a functional gene) together with the Not I restriction enzyme sites (N) based on a published map of the U15 and U10 cosmids [Spies, T. et al., Nature, 348, 744-747(1990)]. The positions of RING 9 and 10 were determined by sequencing of genomic DNA. Mouse cosmid 5.9, which contains the murine transporter genes, also strongly hydridises to the RING 10 probe under non-stringent conditions.

In Fig 2, the sequence is given of the longest RING 4 cDNA clone from the gamma interferon induced U937 cDNA library, p21U, sequenced using the chain termination method [Sanger, F et al., Proc. Nat. Acad. Sci. USA, 74, 5463-5467 (1977)]. The cDNA library was in a derivative of the CDM8 vector and was prepared as described by Seed, B., Nature, 329, 840-842(1987). The open reading frame is shown encoding a protein of up to 808 amino acids. The first and second ATG(AUG) residues are arrowed. The first ATG is immediately preceded by an in-frame termination codon. The second may

correspond to the first methionine in the encoded protein since it is more similar to the consensus sequence of a eukaryotic translation initiation sequence $CC^{A}/_{G}CCAUGG(G)$ [Kozak, M., Nucleic Acids Res., 12, 857-872(1984)]. The vertical line is at the 5' end of the cDNA clone and the additional 5' sequence is obtained from genomic DNA so it remains to be determined if this is contiguous with mRNA sequence. The first AUG is followed by a potential hydrophobic signal sequence. Potential membrane spanning regions are overlined and a region which is highly homologous to the nucleotide-binding regions of related proteins is boxed (see Fig 8). Consensus N glycosylation sites are marked by a dot. The square bracket at position 1838 indicates 3G residues. The p21U cDNA only contained 2 such residues at this position, causing an obvious deleterious frame-shift. To confirm this 3 cDNA's from a B lymphoblastoid cell line, JY and genomic DNA from the cosmid U15 (Fig.1) were sequenced. All of these sequences contained 3 G residues and identical sequences flanking position 1838. It has not been established whether U937 DNA has a similar frameshift in its RING4 gene or whether it is the result of a cDNA cloning artefact. Multiple cDNA clones similar to p21U were obtained but one clone had an insert of several hundred base pairs (bp) at position 1776 due to differential splicing or failure to remove introns.

In Fig.3 the sequence is given of the longest RING 10

clone from a cDNA library made from the T-cell line CEM which was sequenced using the chain-termination method. The cDNA library was in a derivative of the CDM8 vector and was prepared as described by Seed. The open reading frame is shown encoding a protein of 272 amino acids (single-letter amino-acid code). Arrowheads indicate the potential initiating methionines. The predicted amino-acid sequence from the first AUG is shown although the context of this codon does not match the Kozak eukaryotic translation initiation sequence ($CC^{A}/_{G}CCAUGG(G)$) particularly well. The second AUG is in slightly better context and may, in fact, represent the true N terminus of the protein (see text and Fig.14) The consensus sequence for an active-site histidine from the subtilisin family of serine proteases is underlined. The sequence is truncated at the 3' end and contains a further 124 nucleotides before the poly(A) tail.

In Fig. 4 the sequence is given of the longest RING 11 clone obtained from the CEM library is shown. The open reading frame encodes a protein of 686 amino acids. A stop codon (*) is shown just before the potential initiating methionine. Fig.22 gives the sequence of defective mutant RING 11 gene.

In Fig.5 the sequence is given of a cDNA clone of RING 12. The derived amino acid sequence of the longest open reading frame is shown in single letter code above the sequence. The 715bp insert has a 15bp 5' untranslated region followed by a

single long open reading frame encoding 219 amino acids. The TGA stop codon (*) was followed by a 33bp 3' untranslated domain containing a AATAAA polyA addition sequence (underlined). One polymorphic difference between the cDNA and genomic sequence from the U15 cosmid revealed a A-G change (bp194) resulting in a His Arg amino acid change at position 60.

The present invention provides a double stranded DNA containing the coding sequence of at least one of RINGs 4, 10, 11 and 12.

Preferably the double stranded DNA contains the coding sequences of both RINGS 4 and 11 and/or the coding sequence of one or both of RINGS 10 and 12. In one embodiment the double stranded DNA contains the coding sequences for all four of RINGS 4, 10, 11 and 12.

Preferably the double stranded DNA also contains regulatory elements in appropriate location, orientation and where necessary, in reading frame register with the coding sequence of at least one of RINGs 4, 10, 11 and 12. More preferably the double stranded DNA contains regulatory and coding sequences capable of directing expression of at least one of the RING 4, 10, 11 and 12 subunits in a prokaryotic or eukaryotic expression system. Most preferably the double stranded DNA of the invention contains regulatory and coding sequences for all four of RINGS 4, 10, 11 and 12 arranged in the same order and orientation and preferably also having the

same intervening sequences as found in the human MHC complex; such double stranded DNA is, of course, provided in substantially pure form or is present in a cloning or expression vector or as a heterologous insert in a cell.

The present invention also provides products associated with one or more of the genes RING 4, 10, 11 and 12 in the sense that such products embody sequence or structural information derived from the coding sequence of one or other of RINGS 4, 10, 11 and 12. Such associated products include nucleic acids encoding proteins or peptides having a sequence the same as or homologous to at least one of the RING 4, 10, 11 or 12 subunits, nucleic acids encoding proteins or peptides which are capable of interrupting the association of RING 4 and/or RING 11 subunits to form transporter proteins, nucleic acids encoding proteins or polypeptides which are capable of interrupting the association of RING 10 and/or RING 12 subunits with other components of the proteasome complex, nucleic acids which encode proteins or peptides capable of preventing peptide transport by RING 4/RING 11 transporter proteins or capable of preventing proteolysis by the proteasome complex by competitive reversible or irreversible binding to any of RING 4, 10, 11 and 12 subunits, nucleic acids encoding proteins or peptides which bear an epitope recognised by antibodies capable of specifically recognising one or other of the RING 4, 10, 11 and 12 subunits (including nucleic acids encoding mimetopes

of the RING 4, 10, 11 and 12 subunits) and nucleic acids encoding antibodies capable of specifically recognising one or other of the RING 4, 10, 11 and 12 subunits, epitopes thereof, mimetopes thereof or proteins or peptides capable of interrupting association or preventing transport or proteolysis as described below; proteins and peptides having a sequence the same as or homologous to that of at least one of the RING 4, 10, 11 and 12 subunits, proteins or peptides capable of interrupting the association of RING 4 and/or RING 11 subunits to form transporter proteins, proteins or peptides capable of interrupting the association of RING 10 and/or RING 12 subunits with other components of the proteasome complex, proteins or peptides capable of preventing peptide transport by RING 4/RING 11 transporter proteins or capable of preventing proteolysis by the proteasome complex by competitive reversible or irreversible binding to any of RING 4, 10, 11 and 12 subunits and proteins and peptides which bear an epitope (including mimetopes) recognised by antibodies capable of specifically recognising one or other of the RING 4, 10, 11 and 12 subunits; antibodies capable of specifically recognising one or other of the RING 4, 10, 11 and 12 subunits, epitopes thereof, mimetopes thereof or proteins or peptides capable of interrupting association or preventing transport or proteolysis as mentioned above; cells capable of secreting antibodies which specifically recognise one or other of the

RING 4, 10, 11 and 12 subunits, epitopes thereof, mimetopes thereof or proteins or peptides capable of interrupting association or preventing transport or proteolysis as mentioned above; cloning vectors, expression vectors, viral genomes, viruses, transfected prokaryotic and eukaryotic cells and transgenic animals containing exogenous nucleic acid as described above; compounds and compositions which enhance or diminish antigen presentation in association with MHC Class I molecules and processes for identifying such compounds and compositions; pharmaceutical formulations comprising a diluent or carrier and a nucleic acid, protein, peptide, antibody, antibody-secreting cell, vector, virus genome, virus, transfected cell or transgenic animal as described above.

The present invention also provides a product as described above for use in a method of diagnosis or therapy practised on the human or animal body. The invention further provides a diagnostic or therapeutic method comprising administering an effective, non-toxic amount of a product as described above to a human or animal in need thereof.

The present invention further provides the use of a product as described above in the production of a medicament for use in a method of diagnosis or therapy practised on the human or animal body.

The present invention further provides a diagnostic test or assay comprising the use of a product as described above,

for instance by contacting a sample of body fluid or tissue from a patient with a nucleic acid, protein, peptide or antibody as described above.

The present invention further provides processes for producing the products above by conventional techniques well known in the art of biotechnology.

The above aspects of the invention will be described in further detail below.

Nucleic acids

Except as otherwise required, nucleic acids of the invention may be DNA or RNA. The nucleic acids may be single stranded or double stranded. Double stranded nucleic acids may be blunt ended or have 5' or 3' extensions at one or both ends; they may contain one or more restriction endonuclease recognition and/or cutting sites and one or both ends may be cut ends obtained by restriction endonuclease cutting or designed for ligation. Single stranded DNA may be a template strand or a complementary (non-template) strand. DNA may be cDNA, genomic DNA or synthetic DNA. RNA may be mRNA, sense or antisense RNA.

Nucleic acids of the invention may be produced by de novo synthesis and, if necessary, by assembly of fragments to create longer sequences, or obtained from natural sources such as from human cells or by cloning or amplification of natural or synthetic nucleic acids including by transcription or reverse transcription in vitro or in host cells.

Nucleic acids of the invention are useful in diagnosis for instance as hybridisation probes for following alleles of RING 4, 10, 11 or 12 genes through family trees to identify at risk individuals where particular alleles are associated with particular diseases, or to identify mutated or damaged genes in an individual. Nucleic acids are also useful in gene therapy of patients lacking the functional corresponding gene and in producing cloning or expression vectors, viral

genomes or transfected cells for producing proteins or peptides of the invention.

In a particular aspect the invention relates to nucleic acids having a sequence of at least 17 contiguous nucleotide bases or base pairs corresponding to a sequence of the same length within the sequence set out in any one of Figs.2 to 5 or alleles thereof. Preferably the nucleic acid contains at least 20, more preferably 50, 100 or even 200 or more bases or base pairs in a sequence corresponding to that of any one of Figs.2 to 5. Nucleic acid of a total of 17 bases or base pairs will have a sequence identical to the relevant portion of the sequence of any one of Figs.2 to 5 or alleles thereof or will be exactly complementary thereto. Nucleic acids longer than 17 bases or base pairs may have a sequence exactly the same as or differing from the relevant portion of the sequence any one of Figs. 2 to 5 or alleles thereof in one or more bases or base pairs or complementary to such a sequence. Differences may be by substitution, deletion or insertion but should preserve the reading frame where the nucleic acid is intended to be expressed. Preferably such nucleic acids have at least 75%, more preferably 85%, 90% or even 99% homology with the relevant portion of the sequence of any one of Figs.2 to 5 or alleles thereof.

In another aspect the invention relates to nucleic acids encoding proteins or peptides of the invention by use of alternative codons to those used in the normal RING 4, 10, 11 and 12 genes.

Proteins and Peptides

Proteins and peptides of the present invention may be produced by de novo synthesis or by expression of a suitable nucleic acid in a suitable expression system in vitro or in transfected cells or they may be obtained by extraction from natural sources in which case the proteins will be substantially pure and free from other protein and non-protein material with which they are naturally associated in vivo

The proteins of the invention may be used in diagnosis and therapy and for generating antibodies.

In a particular aspect the invention relates to peptides having a sequence of at least 5 contiguous amino acid residues corresponding to a sequence of the same length within the coding sequence of any one of Figs.2 to 5 or alleles thereof. Peptides will preferably be 7 or even 10, 20, 50, 100 or 200 or more residues in length. Those longer than 5 residues may differ in one or more residues from the sequence of any one of Figs. 2 to 5. Differences may be by substitution deletion or insertion. Peptides preferably have at least 75%, more preferably 85%, 90% or even 99% homology with the relevant portion of the coding sequence of any one of Figs.2 to 5 or alleles thereof.

In another aspect the invention relates to proteins and peptides which contain epitopes corresponding to one or more

epitopes of at least one of the RINGs 4, 10, 11 and 12 gene products, or mimetopes thereof, or which are capable of interrupting association of RING 4 and/or RING 11 subunits to form transporter proteins or of preventing peptide transport thereby, or which are capable of interrupting association of RING 10 and/or RING 12 subunits into the protease complex or of preventing proteolysis of the proteasome by specific reversible or irreversible binding, eg in competition with other subunits and/or substrates. Such proteins and peptides may be identified by appropriate assay techniques, for instance as described below.

Antibodies

The antibodies of the invention may be whole antibodies such as IgM or, preferably IgG; they may be polyclonal or monoclonal. The term "antibody" includes fragments of antibodies containing the antigen-recognition site such as F(ab) and $F(ab')_2$ fragments, single domain antibodies (DAbs), complementarity-determining regions (CDRs) and minimal recognition units (MRUs). The term "antibody" further includes anti-idiotypic antibodies and anti-idiotype-2 antibodies (i.e. antibodies against anti-idiotypic antibodies; anti-idiotypic antibodies are those which recognise the antigen-recognition site of an antibody, anti-idiotypic antibodies therefore mimic the antigen whereas anti-idiotype-2 antibodies mimic the antibody) and fragments,

DAbs, CDRs and MRUs thereof.

Antibodies of the present invention may be obtained by conventional immunization techniques and extracted from the body fluids of immunised animals. Alternatively they may be obtained by culturing antibody secreting cells obtained from such immunised animals, preferably after immortalisation by fusion with myeloma cells and other well known techniques. In another alternative, antibodies may be obtained by expressing genetic material, obtained from such antibody secreting cells, in transfected cells.

The antibodies of the present invention are useful in diagnostic tests such as assays performed in vitro and in labelling techniques performed on the human or animal body for diagnosis. The antibodies are also useful in therapy as agents for preventing association of RING 4 and 11 or RING 10 and 12 subunits into transporter proteins or proteasome complexes or in preventing transport or proteolysis by such proteins or complexes.

Cells capable of secreting antibodies

The invention provides cells which secrete antibodies as defined above. Such cells may be obtained by conventional techniques and include antibody-secreting cells obtained from animals immunised against an appropriate antigen, antibody-secreting cells obtained from immunised animals and immortalised by fusion with myeloma cells to form hybridomas,

by use of Epstein Barr virus and other known immortalising techniques and cells capable of expressing and, preferably, processing antibodies on the basis of exogenous nucleic acid inserts.

Cells capable of secreting antibodies as described above are useful as sources of the antibodies and may also be used as diagnostic reagents or as therapeutic agents, for instance by implanting such cells in a patient in order to provide prolonged release of the secreted antibody.

Vectors and expression systems

Cloning vectors, expression vectors, viral genomes (and virus particles containing such genomes), transfected prokaryotic and eukaryotic cells and transgenic animals of the invention all contain exogenous nucleic acids of the invention and may be produced by conventional techniques.

These vectors and expression systems may variously be used for amplification of nucleic acids of the invention, as diagnostic reagents or therapeutic agents and as sources of materials such as nucleic acids, proteins or peptides and antibodies which are themselves products of the invention.

Expression systems such as phage expression systems are particularly useful for raising antibodies against proteins or peptides of the invention expressed at the surface of the host cells.

Labelling

For *in vitro* and *in vivo* diagnostic procedures and for other reasons well known to those skilled in the art it is often convenient to provide products such as the nucleic acids, proteins, peptides and antibodies of the invention with detectable labels. Such labels, for instance radio isotopes, fluorescent chromophores, enzymes, metal particles and polyester beads may be bonded to the products of the invention, used and detected by conventional techniques. Labelled products form a particular aspect of the invention.

Targeting

For therapeutic applications and *in vivo* diagnostic procedures and for other reasons well known in the art it is often useful to target a therapeutic or diagnostic moiety such as a cytotoxic drug or a therapeutic or diagnostic radioisotope or a diagnostic label to a particular site using the recognition properties of a targeting entity such as an antibody. Targeted materials in which a therapeutic or diagnostic product as described above is targeted to a site by a targeting entity, or in which a product as described above acts as a targeting entity for a targeted therapeutic or diagnostic moiety, form particular aspects of the invention.

Separations

In many separations techniques, specific interactions between a ligand and a binding receptor are employed, particularly where the binding receptor is attached to a solid support. The products described above may be used as binding receptors and solid supports bearing such products form a further aspect of the invention. Likewise, products of the invention may be separated and purified using binding receptors, for instance on solid supports. The use of solid supports bearing binding receptors which are, or which specifically interact with, products as described above in such separation processes, forms a further aspect of the invention.

Active Compounds and Assays therefor

The compounds and compositions which enhance or diminish antigen presentation are known or new compounds such as small organic molecules and peptides, proteins, antibodies and nucleic acids of the invention and compositions of matter which interfere with the processing and/or presentation of antigenic peptides by cells through the RING 4, RING 11 transporters and/or RING 10 and/or RING 12 proteasome subunits and which are therefore useful in up- or down-regulating the presentation of antigens and corresponding immune response. Such compounds and compositions may be identified by their ability to enhance or diminish antigen presentation by use of suitable assays. Assays used to

identify such compounds and compositions also form an aspect of the invention.

In a particular aspect the invention provides an assay for the active compounds and compositions described above which comprises simultaneously or sequentially in either order:

(i) contacting a cell capable of presenting antigenic peptides with a standard protein or peptide which is presented by the cell as a peptide: MHC Class I complex, and

(ii) contacting the cell with a compound or composition suspected to enhance or diminish antigen presentation

and thereafter assaying the peptide: MHC Class I complex presented by the cell. Assay by Western blotting or immunoassay techniques is preferred. The results are preferably compared with control experiments in which no compound or composition is used.

The cell may be of a type which is naturally capable of presenting antigen in association with MHC Class I molecules or it may be a mutant or transfectant cell in which the antigen processing and/or presenting systems have been manipulated to improve detection of a particular aspect of the activity of the compound or composition under test. In a preferred embodiment the cell is a transfectant cell containing exogenous nucleic acid of the invention able to secure expression of one or more of RINGs 4,10,11 and 12.

Compounds and compositions which are found to enhance

antigen presentation are potentially useful as immunostimulants, whereas those which diminish antigen presentation compared with the level of presentation seen in control experiments, are potentially useful as immunosuppressive agents.

Since the peptide antigens presented by Class I MHC molecules are usually derived from intracellular proteins, step (i) may be conducted by transfecting the cell with nucleic acid encoding the peptide or protein so as to achieve in situ expression of the peptide or protein which is to be presented. Alternatively cells known to take up peptides or proteins from the culture medium may be used. As a further alternative, peptides or proteins may be introduced by electroporation and other known techniques for delivering peptides and proteins to the cytoplasm.

The compounds and compositions under test may be introduced to the cell by normal cell processes from the culture medium, by electroporation or other known techniques for delivering materials to the cytoplasm or by securing synthesis or expression in situ by known techniques as appropriate.

Therapy

Therapeutic techniques using products of the invention rely upon enhancing a patient's compromised immune system, or enhancing the response of a patient with an intact immune

system to an antigen (for instance to improve the immunity stimulated by a vaccine) or diminishing the response of a patient to an antigen associated with an autoimmune disease. Such treatments may be accomplished by administration of a suitable product of the invention. Alternatively they may be accomplished by administering an expressible nucleic acid encoding an appropriate product such that, when expressed in the patient, the product will have the desired therapeutic effect, for instance by administering transformed cells or virus particles containing the appropriate nucleic acid sequences. In a further alternative, gene therapy may be accomplished by administering modified or transformed bone marrow stem cells having enhanced or inhibited antigen-presenting capabilities, for instance due to manipulation of the genes of the cells encoding any one or more of RING 4, 10, 11 and 12 or by insertion of exogenous nucleic acid of the invention.

Particular therapeutic objectives include enhancement of antigen presentation for instance to improve the immune protection achieved by conventional immunization techniques such as in immunising against influenza. Another objective is in inhibiting either or both the processing of protein antigens and transportation of antigenic peptides in order to diminish antigen presentation in cases where this is required to treat or prevent autoimmune diseases and other immune system disorders.

Diagnosis

Diagnostic processes of the invention include all conventional techniques practised in vitro including nucleic acid hybridisations and immunoassays based on the use of antibodies or proteins or peptides of the invention including direct or competitive ELISA and RIA and fluorescent assays.

The RING4 and RING11 genes are both polymorphic. For example, there are frequent RING11 alleles with differences in: 1) The length of the protein. 2) An amino acid change in the ATP binding cassette and 3) An amino acid change in a transmembrane region. It is believed that these changes reflect differences in the function of the transporters and thus influence the quality of peptides that go towards assembly of Class I molecules in the endoplasmic reticulum. Variations in the proteasome (LMP) genes may have similar affects. These variations may therefore associate with various MHC-linked diseases.

It has been found that certain alleles of RING 11 are associated with particular hereditary immune system disorders such as ankylosing spondylitis and RING 11-related products may be used in pre-natal diagnosis of susceptibility of this disease.

In another aspect the invention provides a diagnostic test kit comprising at least one diagnostic material selected from the products described above.

The invention will now be illustrated by the following Examples which are not intended to limit the scope in any way.

EXAMPLE 1: RING 4

The characteristic feature of the presentation of cytoplasmic antigens, such as influenza nucleoprotein, to Class I restricted T cells, is that the molecules presented lack hydrophobic signal sequences. Deletion of the N-terminal signal sequence of the influenza hemagglutinin can actually enhance presentation in certain conditions. A second feature is that rapid degradation of cytoplasmic antigens is associated with efficient presentation. This suggests that degradation to peptides may occur in the cytosol prior to transport across the membrane of the ER. Consistent with this concept is the finding that short hydrophilic peptides expressed in the cytosol are presented efficiently to T cells.

These features imply that a specialised peptide transport system may exist that passes the peptide products of proteolysis from the cytosol into the ER. Recent evidence shows that association of peptides with the binding site on the Class I heavy chain may be required for the assembly of stable Class I molecules. The mutant cells RMA-S and 721.174 express unstable Class I molecules, are unable to present cytoplasmic antigens but do present extracellular peptides.

This phenotype is consistent with loss of peptide transport from the cytosol into the ER, and in the case of 721.174 is associated with a deletion within the MHC between the DPA2 gene and the complement cluster.

Experiments in the inventors laboratory have been directed towards isolating novel genes in the Class II region of the human MHC. This has been achieved by preparing probes from genomic clones (either cosmids or, more recently, yeast artificial chromosome (YAC) clones) from the MHC and probing these onto cDNA libraries. The initial experiments concentrated on making probes from regions of genomic DNA containing clusters of restriction sites with CpG dinucleotides in their recognition sequence as these are often found near the 5' ends of genes.

The position of the gene RING 4 in the MHC is shown in Fig. 1. It lies at a CpG island which includes recognition sites for the enzyme NotI between the HLA-DNA and -DOB genes, about 25 kb centromeric to the latter.

Short probes were prepared from a 9kb NotI fragment and used to screen cDNA libraries made from mRNA from a B lymphoblastoid cell line, JY, and a gamma-interferon stimulated monocyte cell line, U937. Several hybridising cDNA clones were isolated and the longest was sequenced. The derived amino acid sequencing revealed a long open reading frame and partial sequencing of the NotI fragment used to derive the probes confirmed that it contained the RING4 gene.

Northern blots were obtained as follows:

Total RNA was prepared from the following cell lines; 1. Raji (B lymphoblastoid cell line). 2. Molt 4, T cell line, 3, SW1222 (colon carcinoma cell line). 4. As 3, induced with gamma interferon. 5. SV80 (colon carcinoma cell line). 6. As 5, induced with gamma interferon. 7. DX3 (melanoma cell line). RNA was prepared and gels were run and blotted according to standard protocols (Ausabel, F.M. et al, Current Protocols in Molecular Biology; Wiley Interscience, New York, 1987]. Interferon treatment was growth in 300U gamma-interferon/ml for 36-48h before RNA extraction. A short cDNA clone (p1.2U) was used as a probe. This clone spanned the 1000bp at the 3' end of p21U (Fig.2). Autoradiography was carried out for 3 days using Kodak XAR-5 film.

The blots showed that a mRNA of appropriate length, about 2.8 kb, was expressed in B cell lines and at a lower level in T cell lines (Fig.6). RING4 mRNA was induced also in colon carcinoma cell lines treated with either gamma or α interferon (Fig.6) as well as in melanoma cell line, DX3, that expresses HLA-D region products (Fig.6). Primer extension analysis showed that the longest RING4 cDNA was about 300bp short of the 5' end of the mRNA.

The sequence of the longest clone obtained from the U937 library is shown in Fig.2. The derived amino acid sequence revealed a protein which contains multiple blocks of hydrophobic amino acids characteristic of transmembrane

regions (Fig.7). The data were generated using computer program written by M. Sternberg [CABIOS,7, 257-260(1991)] to use the algorithm of Rao and Argos to predict the location of membrane-spanning regions. The algorithm employs propensities for residues to be within a transmembrane region based on their observed frequency in such regions of several proteins. Regions favourable as potential membrane traversing sequences are marked with horizontal bars and correspond to those pointed out in Fig.2. Note the potential short hydrophobic signal sequence.

Comparison of the RING4 protein sequence with sequences in the EMBL database revealed that it belongs to the ABC superfamily of transporters, which includes the human and mouse multidrug-resistance proteins (MDR) (Fig.8). The protein appears to consist of two distinct domains. The N-terminal domain is very hydrophobic and contains at least six potential membrane spanning α-helices. The C-terminal domain is highly homologous to the ATP binding cassette that is characteristic of this class of transporter. As shown in Fig.8, part of the region of RING4 around the nucleotide binding site is shown aligned with other related proteins from mammals and bacteria: Hmdr, human multidrug resistance gene; Ste6, yeast STE6 gene; OppD and OppF were from S. typhimurium; HlyB is from E. coli; CyaB, CyaB protein from Bordetella pertussis. The region shown is boxed in Fig 2 with two gaps introduced in Fig. 8. These alignments are not

necessarily the best for any two proteins from this set and other regions of homology were observed.

The RING4 ATP binding cassette is not significantly more closely related to those members of the family that transport proteins or peptides. However, it will be particularly interesting to compare in detail other regions of RING4 to the OppA gene of the bacterial oligopeptide permease since OppA encodes the peptide binding domain of the bacterial peptide transporter which is known to bind to a wide range of peptide sequences prior to their transport across the bacterial mambrane.

Typically eukaryotic ABC transporters consist of two hydrophobic domains and two ATP binding domains. The RING4 sequence encompasses only one of each unit. It therefore seems likely that RING4 functions either as a homodimer, or as a heterodimer with a related protein. Compared to the other known ABC transporters, RING4 exhibits an extended N-terminal region, the total product consisting of 808 amino acids, assuming the first AUG codon is used (Fig.2). Also untypical is a potential signal peptide (Figs. 2 and 7).

Example 2: RING 11

Isolation of cDNA clones. cDNA libraries were constructed in a derivative of the CDM8 vector as described by Seed. Libraries were screened with fragments of genomic cosmids and secondary screening of positive clones was reformed as previously described [Kendall et al., Nucleic Acids Res., 18, 7251-7257 (1990)]. DNA from positive clones was prepared using standard protocols. The RING 11 cDNA was initially obtained from a library made from the T cell line CEM which was probed with a fragment of cosmid U10. RING 11 is located between the Class II genes DNA and DOB, approximately 7kb telomeric of RING 4 (Fig.1). The sequence of the CEM RING 11 cDNA is shown in Fig.4. The derived peptide sequence of the longest open reading frame is 686 amino acids and may be divided into two regions. The N-terminal part of the molecule consists of multiple blocks of hydrophobic amino-acids which are characteristic of transmembrane regions (Fig.9).

Fig. 9 shows hydrophilicity plots of RING4 and RING11, the sequences being aligned over the G residue of the GKS (or Walker "A") nucleotide binding motif (residue 508 of RING11) and produced using the MacVector programme (IBI) with an amino-acid window of 7. Potential transmembrane regions are marked by horizontal bars, having been identified as described in Example 1.

Following the transmembrane region is a region which is strongly homologous to the ATP-binding domain of other members of the ABC transporter superfamily.

PCR Amplification

Genomic DNA samples (0.1-1ug) were amplified in 100ul reactions containing 700nM of each oligonucleotide primer, 2mM dNTP's, 1 x Taq Polymerase buffer and 1U Taq Polymerase (Promega). Reaction conditions were 95°C for 10min, 40 cycles of 94°C for 1min, 57°C for 2 min, 72°C for 2 min and a final step of 72°C for 10min.

Sequence Analysis

cDNA clones were initially sequenced by the Sanger M13/dideoxynucleotide chain termination method. Subsequently, 20mer oligonucleotide primers were synthesised over the first full length clone at approximately 200 base pair intervals in both directions. These were then used to sequence further cDNA clones. Solid phase DNA sequencing from PCR products was performed according to a method adapted from Hultman, T, et al., Nucleic Acids Res., 17, 4937-4946 (1989). PCR amplication was performed as described above, except that primer concentrations were reduced to 250nM and a biotinylated 5' primer was used. The PCR product was incubated with 200ug of Dynabeads M-280 streptavidin (Dynal, Oslo, Norway) for 30 min at room temperature. DNA was

denatured by washing once with 0.15M NaOH and once with 0.15M NaOH 0.1M NaCl. Beads were then resuspended in 7ul of distilled water and the DNA sequenced.

Comparisons of RING 11 with RING 4 and other members of the ABC transporter family are shown in Figs. 11 and 12 and Table 1.

Fig 11 is a dotplot comparison between the amino-acid sequences of RING 11 and RING 4 obtained using the Similarity Investigation Programme in the Staden package with an odd span length of 11 proportional score of 132 and a gap penalty of 10. Homology is greatest over the C-terminus ATP-binding domains. Fig. 12 shows a comparison of the ATP-binding domain of RING 11 with RING4, HAM1, MTP1 (respectively, the murine and rat homologues of RING4) and the N-terminus ATP-binding domain of the human multidrug resistance gene product (Hmdr). The single letter amino-acid code is used. The symbol (-) represents an identical residue; (*) represents a gap which has been introduced to optimise the alignment. The Walker A (WA) and B (WB) nucleotide binding motifs are indicated.

Table 1. Table of identity between RING11, RING4 and other members of the ABC transporter superfamily

	RING11	RING4	Hmdr N-terminus	Hmdr C-terminus	HlyB	OppB	OppC	OppD
RING11		29.8	19.2	21.6	18.5	21.0	19.6	-
RING4	61.3		21.1	22.2	16.8	16.9	23.0	-
Hmdr N-terminus	44.4	42.5		30.0	21.7	21.3	19.0	-
Hmdr C-terminus	49.3	45.0	60.2		19.2	16.1	16.1	-
HlyB	43.3	41.2	50.5	47.7		19.7	17.1	-
OppB	-	-	-	-	-		26.6	-
OppD	27.1	29.2	26.5	26.9	29.5	-	-	
OppF	26.6	30.7	31.4	27.4	32.7	-	-	40.3

Table 1 shows identity between RING 11, RING4 and other members of the ABC transporter superfamily. The percent identity was calculated after alignment of sequences using the gene analysis programme in the WUGCG package and a representative sample is shown. For this analysis, polypeptides were split into individual hydrophobic and ATP-binding domains. The Hmdr gene product was split into four as the entire transporter is present on a single polypeptide. Only a single ATP-binding domain and a single hydrophobic domain of HylB (the E. coli ABC transporter for haemolysin toxin) are shown as it is believed this transporter functions as a homodimer. In the upper right-half of the table the hydrophobic domain sequences compared were RING11 (1-469), RING4 (1-504), Hmdr N-terminus (1-399), Hmdr C-terminus (639-1036), HylB (1-459) and the entire OppB and OppC sequences. In the lower left part of the table the ATP-binding domain sequences compared were RING11 (470-686), RING4 (505-748), Hmdr N-terminus (395-639), Hmdr C-terminus (1037-end), HylB (460-690) and the entire OppD and OppF sequences.

Typically each ABC transporter requires two hydrophobic domains and two ATP-binding domains. In the oligopermease system, each of the four domains is formed by a separate polypeptide chain, but in other systems, domains are frequently fused together. For example, all four domains of the multidrug resistance gene product (P-glycoprotein) are

formed by a single polypeptide. RING11 and RING4 each consist of one hydrophobic domain and one ATP-binding domain and, as such, are similar in organisation to the white/brown transporter system of drosophila.

In general, sequence homology between the domains of a single ABC transporter is greater than that between the domains of different transporters. ATP-binding domains are usually more homologous than hydrophobic domains. When the ATP-binding domains of RING11 and RING4 are compared with each other, the sequence homology is 61%, whereas when they are compared with the ATP-binding domains of other transporters, the homology falls to below 50%. Similarly, comparison of the hydrophobic domains of RING11 and RING4 reveals 30% homology, whereas comparison of RING11 and RING4 with the hydrophobic domains of other transporters reveals homology of 20% or less. This implies that RING11 and RING4 function as a heterodimer, although direct experimental evidence to support this is awaited. So far, only circumstantial evidence has been provided by the cell line LCL721.134, which is deficient in RING4 and shows abnormal Class I assembly and peptide presentation. This suggests that RING11 does not function as a homodimer, but does not exclude this possibility for RING4.

Consistent with their proposed role in hydrolysis of ATP, it is assumed that the ATP-binding domains of ABC transporters are located entirely on the cytoplasmic side of

cell membranes. This would also be predicted from the hydrophilic nature of the domains, which contain no potential membrane spanning regions. So far, the only direct evidence to support this comes from studies of the OppF polypeptide in the oligopeptide permease system [Gallagher M.P. et al., Eur. J. Biochem., 180, 133-141(1989)]. If this observation is consistent throughout the family, one would expect the putative RING11/RING4 dimer to be positioned in the ER membrane with its ATP-binding domains within the cytoplasm.

Sequence analysis of the hydrophobic domains from different transporters suggests that each domain usually contains six transmembrane regions, although in some cases eight have been predicted. This has now been supported by a limited amount of experimental data. When hydrophilicity plots of RING11 and RING4 are compared, the hydrophobic regions are strikingly similar in shape and position (Fig. 9). At least six potential transmembrane regions are predicted in each hydrophobic domain, although there may be up to ten in RING4. Elucidation of the exact transmembrane organisation of a putative RING4/RING11 dimer requires further experimentation.

Ring 11 Polymorphism

In order to determine whether RING11 was polymorphic, cDNA clones from two additional B lymphoblastoid cell lines were sequenced. One sequence revealed a single base pair

substitution of T for G at position 1158. This did not lead to an amino-acid change and has not been investigated further. The other cDNA sequence revealed three single base pair substitutions in the 3' region of the gene (Fig.13). The symbol (-) represents an identical nucleotide or amino-acid and the single letter amino acid code is used. Additional variation was also found in the 3' untranslated region of the cDNA clones (data not shown). Two of the coding sequence substituents resulted in amino-acid substitutions, one of which changed the putative stop codon and lengthened the protein by seventeen amino acids. The allele derived from the CEM library, encloding the shorter protein was named RING11A, and the longer allele, RING11B. To characterise these two alleles further, oligonucleotide primers were constructed either side of a 150 base pair stretch of DNA containing the observed polymorphism. The PCR products amplified from the genomic DNA of a number of homozygous HLA typing cell (HTC) lines and a panel of random normal Caucasoid controls were analysed. Allele frequencies shown in Table 2 below, were determined from the PCR products of an HLA homozygous typing cell (HTC) line panel and a panel of normal, Caucasoid control individuals.

Table 2. Frequencies of *RING11A* and *RING11B* alleles

	HTCs (N=36)	Caucasoids (N=54)
RING11A	60/72 (83%)	85/108 (79%)
RING11B	12/72 (17%)	23/108 (21%)

The oligonucleotide primers used to amplify the polymorphic region were GGGGATCGCACAGTGCTGGTG and CTGGAATTCAGGAACAGCTAT. HTCs were analysed using direct, solid-phase sequencing. No other polymorphism was observed using this method. Caucasoid controls were analysed by oligonucleotide typing, using the following pairs of ligonucleotide probes: AGGCTGCAGACAGTTCAG and AGGCTGCAGGCAGTTCAG; CCTCCTAGAGCTGGGCAA and CCTCCTGGAGCTGGGCAA; ATTCCCGCCTGGTGCAGC and ATTCCCCGCCTGGTTCAGC. Note that the frequencies determined for RING 11A account for two nucleotide sequences which differ at positions 489 and 1158, but have identical derived amino-acid sequences.

Class I and Class II MHC antigens are one of the most polymorphic family or proteins known and it is intriguing that a putative peptide transporter located within the Class II region also exhibits polymorphism. So far only a small number of full length RING11 cDNA clones have been sequenced and it is possible that other polymorphisms exist. Nevertheless, two major allelic variants of RING11 have already been identified. At present it is not known whether these alleles are functionally polymorphic with respect to peptide transport, although experiments are currently under way to investigate this. Experimental evidence from other transporter systems suggests that substrate specificity is conferred by the hydrophobic domains rather than the ATP-binding domains. However, it is possible that mutations

within the ATP-binding domains, such as those observed in the cystic fibrosis gene, may also affect the function of transporters. Two independent structural models of the ATP-binding cassette have already been published, based on similarities with adenyl kinase. In both of these, the extreme C-terminus of the domain, in which the RING11A/11B polymorphism is observed, lies outwith the structurally conserved regions. The function of this part of the molecule is thus not known. However, it is of interest that RING11A has one of the shortest C-terminus regions within the transporter family.

An association between MHC antigens and various diseases, such as diabetes mellitus and coeliac disease, is known. As many of the genes within the MHC are in linkage disequilibrium, the possibility arises that some of these associations may primarily be with previously unknown MHC genes such as RING11 and RING4. The region between DNA and DOB is proving to be densely populated with genes. In addition to RING11 and RING4, two of these are thought to be involved in the degradation of protein antigens into small peptides prior to transportation into the ER (RINGs10 and 12).

Oligonucleotide Typing

Typing was performed according to the method of Bugawan et al. [Immunogenetics, 32, 231-241, (1990)] with modifications.

PCR products, immobilised on Hybond N+ membranes (Amersham), were hybridized with biotinylated oligonucleotide probes at a temperature of 56°C. Unbound probe was removed by washing at 59°C.

Northern Analysis

Total cellular RNA was prepared by acid phenol extraction and transferred to Hybond-N membrane (Amersham International) using standard protocols. Hybridization was performed overnight at 42°C and membranes washed at high stringency (0.1 X SSC, 1% SDS, 65°C). Autoradiography was for 16 hours at -70°C using XAR-5 film (Kodak).

On Northern analysis, RING11 was up-regulated by gamma interferon (Fig. 10) an observation common to other genes involved in antigen processing.

In Fig.10, lanes 1-8 show 10 mg of denatured total cellular RNA extracted from normal human keratinocytes after incubation with 200 units per ml of recombinant gamma interferon for 2, 6, 24, 48, 72, 96, 120 and 168 hours respectively; lane 9, 10mg of total cellular RNA from uninduced keratinocytes. The filter was hybridized with the CEM derived RING11 cDNA shown in Fig. 4.

Example 3: RING 10

It is now possible to paint a detailed picture of how cytoplasmic proteins are handled by the immune system. They are apparently degraded in the cytoplasm into peptides. These are then transported into the endoplasmic reticulum where they encounter Class I major histocompatibility complex (MHC) molecules. Once loaded with peptide, the HLA molecules move through the Golgi apparatus to the cell membrane. Until recently, it had not been established how peptides without signal sequences cross the ER membrane. However, Examples 1 and 2 describe a pair of membrane transporter genes of the ABC (ATP-binding cassette) superfamily which apparently serve this function. Both transporter genes, which may encode two halves of a heterodimer, are situated in the Class II region of the MHC. There is evidence that other putative components of the processing machinery, the LMP's (low molecular mass polypeptides), are also encoded in the MHC. Similarities between the properties of the LMPs and a large intracellular protease complex, called proteasome, have led to the suggestion that LMPs are involved in processing antigens. This Examples identifies a human gene with sequence homolgy to proteasome components. Remarkably, this gene maps between the two putative peptide transporter genes.

cDNA libraries derived from a T-cell line, CEM, and a gamma-interferon-stimulated monocyte line U937, were screened with fragments of cosmids U10 and U15. This revealed another

two genes, RING9 and RING10, located between RING4 and RING11 (Fig.1).

The sequence of the longest RING10 cDNA, isolated from the CEM library, is presented in Fig. 2. The sequence contains a long open reading frame which, from the first methionine, is predicted to encode a protein of 272 amino acids, relative molecular mass 30,000 (M_r, 30K) and pI 5.41. There is evidence to favour the second in-frame methionine at amino acid position 65 as the translational start site: first, it matches the Kozak consensus more closely and second, it is only a few residues upstream of a region which shows sequence homology with a number of N-terminal sequences from rat and human proteasomes (see later and Fig.14. This would result in a protein of 208 amino acids, M_r 23K and pI 7.78.

The derived amino acid sequence of RING10 was compared with sequences in the PIR 27, Swiss Prot 17 and OWL databses and a match found with a component of human proteasome. The proteasome (also known as macropain or multicatalytic proteinase complex) is a large intracellular protease. It consists of about 15 polypeptide components, depending on the species. These components range in M_r from 20 to 35K and are ordered into a cylindrical structure of M_r 650K [McGuire, M.J. & DeMartino, G.N. Biochim. biophys. Acta, 873,279-289 (1986); Lee. L.W. et al., Biochim. biophys. Acta, 1037, 178-185 (1990); Rivett. A.J., Archs. Biochem. Biophys., 268, 1-8(1989)]. A very good match was seen with a number of

limited amino-acid sequences, obtained by N-terminal sequencing, from several human and rat proteasome components. Notable among these are human proteasome epsilon chain and rat chain 6 (Fig. 14). Full-length comparisons with these chains await the cloning of their respective cDNA's.

Fig. 14 shows alignments with sequences derived from N-terminal amino acid sequencing of human (HUM epsilon, HUM δ, HUM ß) and rat (RAT 5,6 and 7) proteasome components and cDNA sequencing of human (HUM C3), Drosophila melanogaster (Dm35), Saccharomyces cerevisiae (ScC1) and Thermoplasma acidophilum (TAα) proteasome components. Dashes show positions at which there is identity with RING10, lower case indicates conservative changes [Argos, P. J., Molec. Biol., 193, 385-396(1987). "X" represents a residue for which identification was uncertain. Numbering refers to RING10. The RING10 and human epsilon chain sequences shared 15 out of 22 amino acids plus some conservative substitutions, indicating that the sequences are undoubtedly related. The alignment with cDNA-derived protein sequence is not as striking as that with the human epsilon or RAT 6 peptides. Note that, although the amino acid sequences were obtained from N-terminal sequencing, they align at some distance from the first methionine of RING10. Although this may be due to proteolysis, it is consistent with translation initiating at the second in-frame AUG codon. Low-stringency diagon plots between RING10 and a selection of cDNA sequences from a

variety of species are shown in Fig. 15 and alignments of amino acids 65-94 and 165-187 are shown in Figs. 14 and 16 respectively. Fig. 15 shows diagon plots of cDNA-derived protein sequence from Human C3, Human C5, Dm 35, ScC1 and Taα against RING10 and Human C3 against TAα. The comparisons were run using the Similarity Investigation Programme in the Staden package with a window of 31, a proportional score of 330, and the default score matrix. Note that extrapolation of the plots to the RING10 axis in the first five cases again suggests that the methionine at amino acid 65 is the initiating residue. The sixth diagon shows homology between Human C3 and TAα over the whole protein length for comparison. These two proteins have about 40% identical residues. The full-length human chain sequences show 10-35% identity with each other (data not shown). Fig. 16 gives an alignment of RING10 with a stretch of Human C3, Human C5, Dm 35, ScC1 and TAα cDNA-derived protein sequences. Symbols and numbering are as in Fig.15 This alignment also shows weaker homology than that obtained with the N-terminal peptide sequences.

As the proteasome is thought to be multiproteinase complex with broad specificity, its components should have active sites able to catalyse several proteolytic reactions. A protein motif search of the PROMOT database [Rivett. A.J., J. Biol. Chem., 260,12600-12606(1985)] revealed a good match between RING10 and a consensus sequence derived from over

twenty members of the subtilisin family of serine proteases.

Fig. 17 compares RING10 with serine protease active sites. Symbols and numbering are as in Fig. 15 with the exception that "X" represents any amino acid in the consensus. The serine, histidine and aspartate residues, which may make up the catalytic triad in RING10 are marked with an asterisk. The histidine active-site consensus, from the subtilisin family of serine proteases was identified by screening the RING 10 protein sequence using the programme PROMOT and shows an exact match at amino acids 185-194. The probability of such a match occurring randomly is close to 1 in 7000. At least 20 different sequences from known subtilisin-like serine proteases were used to determine this consensus. Alternative consensus residues are also shown. Suggested alignments, obtained by inspection, of the linear sequences surrounding the potential active site aspartate and serine residues of RING10 with those of known subtilisin-like serine proteases are also shown. The subtilisin BPN' sequence is from Bacillus amyloliquefaciens, subtilisin S from B. subtilis var. amylosaccharitícus, furin is a human protease and KEX2 is from S. cerevisiae.

Known serine protease active sites consist of three regions each containing a residue involved in the catalytic mechanism. Typical consensus sites for the other two residues of the serine protease catalytic triad, aspartate and serine, were not identified by the motif search, but

plausible alignments with known serine proteases could be found by inspection and are shown in Fig.17. The order of these putative catalytic residues in RING10 (serine, histidine, aspartate) differs from that of both subtilisin-like protease (aspartate, histidine, serine) and trypsin-like proteases (histidine, aspartate, serine). Thus, if RING10 is a serine protease, it is atypical and a definitive assignment of its function requires biochemical analysis.

The LMPs were originally defined by two-dimensional PAGE of immunoprecipitates obtained from a mouse macrophage line. [Monaco, J.J. & McDevitt, H. O., Proc. Natl. Acad. Sci. USA., 79,3001-3005 (1982); Nature, 309, 797-799 (1984) and Human Immun., 15,416-426(1986)]. They consist of 15 polypeptides with M_rs of 15-30K which noncovalently associate to form a complex of 580K. The complex resembles proteasome [Parham, P., Nature, 348, 674-675 (1990)], indicating that LMP's might be identical or related to proteasome components. Using recombinant strains, the genes for two of the LMP's have been localized to a region of the mouse MHC between Pb and Ob, the equivalents of DP and DOB in the human (see Fig. 1). It is possible that other LMP genes are also located in the MHC but, due to a lack of detectable polymorphism, have not been mapped with this method. It has been proposed that LMP's might be involved in antigen processing, degrading cytoplasmic proteins into antigenic peptides before their transport into the ER by the MHC-linked transporters.

Consistent with this role is the increased expression of LMP proteins in the presence of gamma-interferon, a property shared with Class I and II MHC antigens and the putative peptide transporters. Expression of RING10 is also induced by gamma-interferon as shown by Fig. 18.

Fig. 18 shows Northern blots as follows: a, Lanes 1, T-cell line (J6), 2, B-cell line (MANN) and 3, Human monocyte cell line (U937). b, Normal human keratinocytes grown in 200 U recombinant human gamma-interferon ml^{-1} for 2, 48, 72 and 96h before RNA extraction (20 μg total cellular RNA was run per (track). The first lane shows RNA from untreated cells. The cDNA clone shown in Fig.3 was used as a probe. Autoradiography was for 24 h using Kodak XAR-5 film. Standard protocols were used.

The exact relationship between proteasome, LMP's and RING10 remains to be established. But the finding of such an intimate association between a proteasome gene and the two transporter loci implies a close relationship at the regulatory or functional level.

EXAMPLE 4: RING 12

MHC Class I molecules present endogenous peptide to CD8+ cytotoxic T lymphocytes. Peptides derived by degradation of antigenic proteins in the cytosol are thought to be transported into the endoplasmic reticulum (ER) where they participate in the assembly of Class I molecules. This Example relates to the identification and characterisation of a second gene with homology to proteasome components in the human MHC. This gene may share a close evolutionary ancestry with RING 10.

Total B-lymphoblastoid cell mRNA was isolated by lysis in guanidinium isothiocyanate and purified by cetrifugation in caesium chloride and used to prepare a NBC cDNA library in the cDM8 vector. The library was plated and screened on HybondN+ (Amersham) as described by the manufacturers using fragments of genomic DNA from cosmid U15, isolated in low melting point agarose, as probes (labelled probes were competed with human DNA prior to hybridisation) and the RING 12 clone was isolated. The cDNA probe was then hybridised back to the cosmid to map the position of the RING12 gene. The complete genomic sequence of the region was also determined to facilitate this. The RING 12 gene lay, 5' to 5' about 400bp from the RING4 gene. High stringency hybridization of the cDNA probe to human genomic DNA on Southern blots showed that RING12 was a single copy gene.

The expression pattern of RING 12 was determined by Northern blotting of RING 12 MRNA. The 900bp band observed (Fig.19) corresponds to the RING 12 transcript and the higher MW band is from a previous hybridisation with the 2.5kb transporter cDNA, RING 11. Colon carcinoma (tracks 1 and 2 CC20, 3 and 4-SW620, 5 and 6-SW1222, 7 and 8-PAF), and SV40 transformed fibroblast cell line samples (tracks 9 and 10-SV80, 11 and 12MRC5SV), Molt4 T cell (track 13) and Raji, B cell line (track 14), were loaded with 15μg total RNA each in the even numbered lanes 2 to 12, cells were treated for 48hrs with 250 units/ml of IFN-gamma. Ring 12 detected a 900bp transcript in both B and T lymphocytes. Expression was strongly upregulated by IFN-gamma in epithelial (colon carcinoma) and fibroblast cells lines. Basal mRNA levels were barely detectable in the colon carcinoma lines. This was in contrast to two SV40 transformed lines where expression was apparent prior to IFN-gamma induction.

The RING 12 cDNA insert was sequenced by the chain termination method using the random dideoxy nucleotide approach and sequences were assembled using the SAP program. The 715 bp insert contained a single long open reading frame encoding a 219 amino-acid product (Fig.5), with a predicted molecular weight of 23.2Kd and a pI of 4.64. When compared with the full genomic sequence, a single base pair difference was observed (G for A bp194. Fig.5) which resulted in the substitution of a histidine for an arginine residue (aa60).

Several lines of evidence suggest that the protein shown is a full length product. First, the cDNA clone is approximately the same size as products detected on Northern blots and second, although the initiating AUG codon is not preceded by a stop codon, 5' genomic sequence data shows no further AUG codons before reaching the adjacent gene, RING 4. Finally, although the predicted initiating Met codon conforms only poorly to the eukaryotic translational initiation sequence consensus, the N-terminal amino acid sequence matches limited peptide data available from related proteins.

By searching protein databases (Swiss Prot 17 and PIR27) the RING 12 protein was found to be homologous to fragments of N-terminal amino-acid sequence from rat and human proteasome components (Fig.20). In Fig. 20, identical residues a marked "-", "X" denotes an unknown residue. Only partial amino acid sequence data is available for some rat chains (1 to 7) and human proteasome subunits (chains A to E). Proteasome components have molecular weights in the range of 20-35 kD for instance about 23 kD and pIs of between 4.5 and 7.5, consistent with the RING 12 amino acid sequence. With the limited sequence data available, RING 12 appears most similar to rat chain 7 (19 out of 21 amino acids). These proteins also appear most similar in molecular weight. The mouse LMP2 protein, which was mapped to the H-2 region on the basis of its polymorphism, has a similar molecular weight and pI to RING 12. Comparison of RING12 with other complete

human sequences showed greatest identity to RING 10 (30%) and 17-23% identity with other proteasome sequences (Table 3) in which amino acid identity amongst human proteasome genes is calculated as a percentage. Alignments were performed using the default parameters in the GAP programme (GCG).

Table 3

TABLE OF % AMINO ACID IDENTITY BETWEEN HUMAN PROTEASOME GENES

	R12	R10	HC2	HC3	HC5	HC8	HC9
R12							
R10	30						
HC2	17.8	19.4					
HC3	20.4	24.1	31.8				
HC5	23.6	21.6	18.1	21.1			
HC8	19.5	17.3	26.8	30.6	15.7		
HC9	17.5	17	29.5	37.8	23.3	30.5	

Unlike RING 10, RING 12 showed no obvious homology to protease active site motifs in the PROMOT database. The proposed RING 12 sequence is at least 18 amino acids longer at the N-terminus than published peptide fragment sequences.

It is remarkable that a second proteasome gene should lie so close to RING 10 and the ABC transporters genes, RING 4 and RING 11. It is now well established that the MHC contains several clusters of genes with related immunological functions, but it is not apparent why they should be tightly linked. One possibility is that genes are clustered because of coordinated regulation. The promoters of the RING 12 and RING 4 genes, for example, are within a few hundred base pairs of each other (the first exon of RING 12 maps less than 400 bp upstream of the RING 4 gene) and may share an interferon response element. Alternatively, genes may be linked because of a selective advantage in maintaining certain combinations of polymorphic proteins together. This may help to explain the occurrence of extended haplotypes and their complex association with disease.

Duplication is a hallmark of the MHC which has examples in Class I and Class II genes, TNFA and B, HSP70-1 and C4A and C4B. RING 12 and RING 10 amino acid sequences are closer to each other than they are to other human proteasome sequences, suggesting that they arose by gene duplication. The duplication unit may have included an initial transporter gene associated with a proteasome component, which gave rise

to RING 4, and RING 11 at the same time as RING 10 and RING 12. It is not a simple duplication, line DQA and B, because RING 12 and RING 4 are arranged head to head and RING 10 and RING 11, tail to head which implies a simple inversion of one or more of the genes though a more complex arrangement is also plausible.

The discovery of a second proteasome-like gene in the Class II region of the MHC provides further evidence that proteasomes are involved in antigen processing, as outlined in Fig.26. But, whereas classic proteasome components are expressed constitutively and constitute a major fraction of total cell protein, RING 10 and 12 are expressed at a very low level in some cells. It is proposed that the RING 10 and 12 proteins are proteasome-related components specialised for antigen processing. Intuitively, it seems likely that antigen processing may use a combination of housekeeping proteolytic functions and specialised recruiting factors such as RING 10 and 12. Mutants can be used to investigate this.

Fig.26 illustrates the proposed involvement of RINGs 4, 10, 11 and 12 in protein antigen processing and presentation. Proteasome components RING 10 and 12 are shown involved in the degradation of endogenous protein. Resulting antigenic peptides are subsequently transported from the cytosol into the endoplasmic reticulum via the RING 11 gene products. Mapping and sequencing of cosmid U15 showed that the RING 4 (transporter) and RING 12 genes are on opposite DNA strands

and their 5' ends are less than 400 bp apart.

Example 5: RING 4/11 HETERODIMERS

This Example shows that the proteins encoded by RING 4 and 11 genes form a complex that may fulfil the role of peptide transporter. Loss or mutation of either protein results in a cell phenotype characterised by the formation of unstable Class I molecules, with loss of presentation of intracellular antigens to Class I restricted cytotoxic T cells.

The following figures are referred to in this Example:-

Fig.21: This shows the detection of the RING4/11 protein complex with the AK1.6 serum to the RING4 polypeptide. Fig. 21 (a) shows immunoprecipitates prepared from the following cell lines : lane 1, the mutant .174; lane 2, the wild type cell 721; lane 3, BM36.1; lane 4, BM28.7; lanes 5 and 6, independent populations of BM36.1 cells transfected with a RING11A cDNA; lane 7, T2 cells; lane 8, T2 cells transfected with a RING11A cDNA. The ~71KD, ~77KD and ~83KD bands are marked. Fig.21(b) shows immunoprecipitates from a series of cell lines genotyped for their RING11 alleles (shown in brackets). Lane 1, LBL721 (A,B); lane 2, BM.28.7 (A,-); Lane 3, C1R (A+/-, B, see text) ; Lane 4, PRIESS (A,B); Lane 5, STEINLIN (A,B); Lane 6, HOM-2 (A,B); Lane 7, BASILIO (B,B); Lane 8, COX (A,A); Lane 9, P.H. (A,B); Lane 10 P.W. (B,B);

Methods: The antiserum to RING4 (AK1.6) was raised by a standard procedure. A Sandy half-lop rabbit was immunised with 0.5mg of the peptide GCYWAMVQAPADAPE glutaraldehyde cross-linked to keyhole limpet haemocyanin (KLH) in complete Freund's adjuvant, and boosted at 20 day intervals. The AK1.6 serum was taken after the 5th injection of antigen. Immunoprecipitation from aliquots of $2x10^7$ cells was done as described by Townsend, A.R.M. et al., Cell, 42, 457-467 (1985). Townsend,A. et al., Nature, 340, 443-448 (1989) and Townsend,A. et al., Cell, 62, 285-295 (1990). Cells were labelled for 2-3hrs with 200 μCi ^{35}S methionine, lysed in 1m of lysis buffer, and the antiserum added to 1:40 dilution. Precipitates were eluted from antibody in reducing conditions and heated for 4 minutes at 95°C before electrophoresis on 10% SDS polyacrylamide gels.

Origins of BM36.1 and BM28.7: The BM28.7 cell (HLA-A1), B35/BW6, Cw4) is a hemizygous irradiation mutant of the wild type EBV transformed lymphoblastoid cell line BJAB-B95.8.6 (Klein, G. et al., Int. J. Cancer, 18, 639-652(1976) and Zeigler, A. et al., Immunobiol., 169, 455-460 (1985). BM36.2 was derived from BM28.7 after fractionated irradiations (5Gy and 2Gy) and selection for BW6 loss with a monoclonal antibody (SFR8-B6, Radka, S.F. et al., J. Immunol. 128, 2804-2806 (1982)) and complement followed by cloning (Spring, B.

et al. Immunogenetics, 21, 277-291(1985) and Zeigler, A. et al., loc. cit.)

Transfection of BM36.1. A sequenced RING 11A clone was subcloned as an XhoI fragment into the episomal expression plasmid pREP9 (Invitrogen), electroporated into BM36.1 cells and then transfected cells were selected in Neomycin. Two independent polyclonal drug resistant cultures were analysed in these experiments as all the cells in the transfected cultures were found to re-express HLA A1 and B35 at the cell surface by indirect immunofluorescence.

Fig.22: This illustrates mutation in the RING11 gene from BM36.1. Fig.22(a) shows Southern analysis of mutant cell lines; Fig.22(b) shows Northern analysis of mRNA from mutant cell lines; lane 1, BM28.7; lane 2, BM36.1; lane 3, T2. Fig 22(c) shows sequence of the mutant RING11 gene from BM36.1.

Methods:

Southern analysis:

Genomic DNA was prepared by salt chloroform extraction. DNA was digested to completion with Hind III, electrophoresed through 0.7% agarose and transferred to Hybond-N membrane (Amersham) using standard protocols. A full length radio-labelled RING11 cDNA was used as a probe. Autoradiography was carried out at -70 °C for 24hrs using XAR5 film (Kodak).

Northern analysis:

Total cellular RNA was prepared by acid phenol extraction, electrophoresed under denaturing conditions and transferred to Hybond-N using a standard protocol. Sequential hybridisations were performed overnight at 42°C, then the membrane was washed at high stringency. Autoradiography was carried out at -70°C for 16 hrs. Radio-labelled RING11, RING4 and GAP-DH were used as probes. GAP-DH is a universally expressed message that served as a positive control.

DNA sequencing: Single strand cDNA was made from polyA purified BM36.1 RNA, using reverse transcriptase and an oligonucleotide primer specific to the non-coding 3' region of RING11. The manufacturer's (Boehringer) protocols were used. Two further RING11 specific oligonucleotides were then used to amplify the entire RING11 cDNA by polymerase chain reaction (PCR). Portions of the PCR product were re-amplified using RING11 specific biotinylated oligonucleotides and solid phase DNA sequencing performed as previously described. The two base pair deletion at position 635, indicated in the figure, was confirmed by solid phase sequencing of BM36.1 genomic DNA. Single letter amino acid code is used.

Fig.23: This shows the effect of transfection with a RING11A cDNA on the stability of Class I molecules expressed in BM36.1. The stability of Class I molecules was assessed by immunoprecipitation with the conformation sensitive monoclonal antibody W6/32 (Barnstaple, C.J. et al., Cell, 14, 9-20(1978)) and Parham, P. et al., J.Immunol., 123, 342-349(1979) from detergent extracts of BM28.7 (lanes 1 - 3); two independent populations of BM36.1 transfected with a RING11A cDNA (lanes 4 - 6, and 7 - 9); or BM36.1 (lanes 10 - 12). The antibody was added either immediately after cell lysis (lanes 1, 4, 7, 10); after overnight incubation of the cell lysates at 4°C to allow dissociation of β-2m (lanes 2, 5, 8, 11); or after addition of unlabelled human β-2m to the lysates to 3μM followed by overnight incubation at 4°C (lanes 3, 6, 9, 12).

Methods:

Immunoprecipitations were done as described by Townsend,A. et al., Nature, 340, 443-448 (1989), Townsend,A. et al., Cell, 62, 285-295 (1990) and Elliott, T.J. et al., Nature, 351, 402-406 (1991) with the following minor modifications: Briefly, 3 aliquots of -10^7 cells from each cell line were internally labelled with ^{35}S methionine, lysed in 1ml , and

the insoluble material removed. Immediately after lysis purified W6/32 antibody was added to one aliquot 15μg/ml (10^{-7}M), human β-2m (Sigma) to a second aliquot to 36μg/ml (3 x 10^{-6}M), and to the third aliquot 25μl of phosphate buffered saline as a control. The lysates were incubated at 4°C overnight, and without any preclearing W6/32 antibody was added to the second and third aliquots to 15μg/ml for 90 mins. The immunoprecipitates were then collected with protein A sepharose beads, eluted, reduced and separated on 12% SDS polyacrylamide gels.

Fig.24: This shows that Class I expression increases in BM36.1 transfected with a RING11A cDNA. Fig.24(a) shows HLA-A1 expression, fig.24(b) shows HLA-B35 expression. The mean fluorescence values for the cell populations are shown in the figure.

Method: Class I expression was measured by indirect immunofluorescence as described by Townsend,A.R.M. et al., Cell, 42, 457-467 (1985). The first layer antibodies were in a) 142.2, specific for HLA-A1 (Smith,M.E.F. et al., Proc. Natl. Acad. Sci. USA, 86, 5557-5561 (1989)) purified at 5μg/ml), and in b) 4D12 specific for members of the HLA-B5 cross-reactive group (Haynes,B.F. et al., Hum. Immunol., 4, 273-285 (1982) (as neat culture s/n). The second layer antibody was fluorescein labelled, affinity purified, goat

anti mouse immunoglobulin (Sigma F0257) at 1:40 dilution.

Fig.25: This shows the presentation of influenza antigens in BM36.1 transfected with a RING11A cDNA. An HLA-A1 restricted cytotoxic T lymphocyte (CTL) line was tested for recognition of the following influenza infected (squares) or uninfected (circles) cell lines: a) autologous EBV-transformed lymphoblastoid cell line; b) BM36.1 transfected with a RING11A cDNA; c) BM36.1; and d) BM28.7.

Method:

Fresh peripheral blood lymphocytes obtained from donor NR (HLA-A1, A3, B8, B60) were stimulated in vitro with influenza AX31 as described by Gotch,F. et al., Nature, 326, 881-882 (1987). After 7 days the cells were restimulated with an HLA-A1 restricted peptide derived from the influenza A nucleoprotein (amino acids 89-101) and after a further 7 days the CTL were tested in ^{51}chromium release assay for recognition of influenza AX31 infected target cells. Spontaneous ^{51}Cr release in the absence of CTL ranged between 22%-35%.

Expression of two allelic forms of a transporter complex

To investigate the expression of the proposed peptide transporter antiserum (AK1.6) was raised by a standard

procedure against a peptide representing the C-terminal 15 amino acids of the RING4 protein sequence. The peptide was chosen partly for lack of similarity to the RING11 sequence. Immunoprecipitation from extracts of the normal EBV-transformed lymphoblastoid cell line LBL721 (Fig.21a, lane 1) revealed two major bands that were absent in extracts from the mutant .174 (Fig.21a, lane 2). As both HLA haplotypes of .174 cells are affected by large deletions which include both of the transporter genes, this result suggested that the two proteins may be encoded by these two loci. The bands migrated with apparent molecular weights of -77KD (upper) and -71KD (lower), fig.21a, lanes 1 and 2).

The -77KD band from extracts of LBL721 resolved into two species, of slightly different size (-1.5KD), when separated on 10% polyacrylamide gels and exposed directly to X-ray film without amplification (Fig.21b, lane 1). The RING11 gene has recently been found to have two alleles, that should encode proteins that differ to length by 17 amino acids. These were referred to as "A" for the shorter and "B" for the longer form. This difference in length would be sufficient to give rise to the slight change in migration seen in the two upper bands isolated from LBL 721. A comparison of the proteins detected with the anti RING4 antiserum from a set of ten cell lines with known RING11 genotypes was made.

Fig.21b shows that all the cell lines tested gave rise to a lower band of -71 KD. However, each cell line had one of three patterns of the upper bank(s); "A" (lanes 2,8), "B" (lanes 7,10), or "A" + "B" (lanes 1, 4, 5, 6, 9). In nine cases the migration patterns of these bands matched the RING11 genotypes, as determined by sequencing of amplified genomic DNA. However, the highly mutated cell line C1R appeared to be homozygous B by sequence analysis, while the immunoprecipitates contained band B, as well as a weak band A (lane 3).

The data from the nine cell lines suggested strongly that the upper band(s) detected in LBL721 by the antiserum to RING4 were encoded by the two alleles of the RING11 gene, and were co-precipitated with the RING4 protein (lower band). The identification of the lower band as the product of the RING4 gene was confirmed by immunoprecipitations from the mutant .134, which lacks only expression of the RING4 mRNA. As expected, neither the upper -77KD (RING11) nor the lower -71KD (RING4) proteins were precipitated from the mutant .134 cells transfected with the RING4 gene. Cross-reaction of the anti RING4 serum with the RING11 protein was ruled out by lack of precipitation of either band from T2 cells (a derivative of .174, Salter, R.D. et al., EMBO, 5, 943-049(1986) transfected with a RING11A gene (Fig.21a, lanes 7 and 8).

The RING11 protein migrated in the SDS gels close to its predicted molecular weight of 75.5 KD. By contrast, RING4 migrated at ~71KD, which is ahead of its predicted molecular weight of 81KD (calculated from the first ATG codon in the cDNA sequence). The reason for this is not clear, however similar aberrant migration in SDS gels of related proteins in the ABS family does occur.

Finally, in some experiments, proteins in the 20KD - 30KD range co-precipitated with transporter proteins from extracts of 721, but not .174. Such a band is visible in lane 2 of Fig.21a. These proteins are in the size range expected for proteosome subunits; proteins in the size range of the transporters were co-precipitated with certain sera to proteosome components.

A mutant cell line with defective RING11 protein

Further experiments with a pair of mutant lymphoblastoid cell lines, BM36.1 and BM28.7, confirmed the identity of the RING11 band and provided evidence for a function of the RING11 protein. These two cell lines were selected sequentially for loss of Class I expression from the heterozygous cell line BJAB-B95.8.6. BM28.7 is hemizygous, having deleted one HLA haplotype, but retains normal expression of Class I molecules encoded by the second

haplotype (HLA A1, B35/Bw6, Cw4). BM36.1 was derived from BM28.7 (see legend to Fig.21), and has a phenotype similar to RMA-S. The Class I molecules synthesised and assembled in BM36.1 are unstable in vitro (Fig.23), are expressed at low levels at the cell surface (Fig.24), and do not present intracellular influenza proteins to HLA A1 restricted cytotoxic T cells (Fig.25).

Immunoprecipitation with the anti RING4 serum from extracts of BM36.1 revealed that the ~77KD RING11 band was missing from this cell line, but was present in the precursor hemizygous cell line BM28.7 in the A form (Fig.21a, lanes 3 and 4; Fig.21b, lane 2). However, a unique band of higher molecular weight (~83KD, marked in Fig.21a) was co-precipitated from extracts of BM36.1. Transfection of BM36.1 with an A allele of RING11 restored the expected RING11A band in immunoprecipitates, with associated loss of the higher molecular weight protein (Fig21a, lanes 5 and 6).

Analysis of genomic DNA by Southern analysis from BM36.1 and BM28.7 revealed no abnormalities in the RING11 gene, and RING11A mRNA expression was indistinguishable in the two cell lines (Fig.22a,b). However, sequence analysis of the RING11 cDNA from BM36.1 revealed a deletion of two base pairs at codon 635, which lies immediately 3' to the Walker B nucleotide binding motif in the RING11A sequence (Fig.22c).

The consequent change in reading frame resulted in replacement of the C-terminal 50 amino acids of the RING11A ATP binding domain with new sequence, and a 50 amino acid extension to the protein. This was consistent with the increase in molecular weight of -6KD seen in the unique protein co-precipitated from BM36.1 with the anti RING4 serum (Fig.21a, lane 3 and Fig.22c).

Reversal of the mutant phenotype by transfection with RING11

The mutant HLA phenotype of BM36.1 was reversed by transfection with a RING11A cDNA clone. The intracellular Class I molecules in BM36.1, like those of RMA-S, are loosely associated with ß2-microglobulin and lose the conformation detected by the antibody W6/32 when diluted in vitro (Fig.23, lane 11). They can be stabilised either by addition of the antibody immediately after cell lysis (Fig.23, lane 10), or by raising the concentration of ß-2m in equilibrium with the heavy chains (Fig.23, lane 12). By contrast the majority of Class I heavy chains released from the hemizygous cell BM28.7 retained the conformation detected by the antibody W6/32 after lysis in vitro (Fig.24, lanes 1 and 2). Transfection of BM36.1 cells with RING11A cDNA resulted in an increase in the proportion of stable Class I heavy chains detected with W6/32 (lanes 4 - 6 and 7 - 9).

Class I expression at the cell surface (Fig.24) was also

restored to -50% the level on the hemizygous precursor BM28.7, (which is indistinguishable from non mutant EBV-transformed cell lines; data not shown). Finally, presentation of influenza antigens to an HLA-A1 restricted cytotoxic T cell line was restored in BM36.1. transfected with the RING11 gene (Fig.25b), to levels comparable to the BM28.7 precursor Fig.25d), or the normal HLA matched lymphoblastoid cell line (Fig.. 25a).

Discussion

These experiments show that the products of the RING4 and 11 genes form a complex that can be detected by co-precipitation in vitro. Loss or malfunction of either RING11 (cell line BM36.1 and RMA-S) or RING4 (Cell line .134) results in the formation of unstable Class I molecules that do not present peptides derived from intracellular antigens to Class I restricted CTL. These properties, and the structural similarities of the RING4 and 11 proteins to other proteins with transport functions, suggest that the RING4/11 complex controls the delivery of peptides to Class I molecules in vivo. Although the stoichiometry of the complex cannot be deduced from these results, they are consistent with the RING4 and 11 proteins forming a heterodimer.

The deletion of two base pairs in the RING11 sequence in BM36.1 occurred immediately 3' to the Walker B nucleotide

binding motif. This mutation resulted in substitution and elongation of the C-terminal region of the ATP-binding domain with novel sequence. The mutant protein was still able to form a complex with RING4, which suggests that the mutated region is not involved in binding to the RING4 protein. The wild-type RING11A protein displaced the mutant form in transfected cells, presumably because the transfected gene was expressed at a high level and competed for binding to RING4. The mutant complex clearly lacked function, possibly through loss of ATP hydrolysing activity or interactions of the ATP binding domain with other, unknown, subunits.

Two alleles of the RING11 gene have been described that differ in the length of their ATP-binding domains by 17 amino acids. Both these alleles of RING11 can form a complex with RING4, and these two forms of RING11 are co-expressed in heterozygotes. It is not yet known whether the polymorphism in RING11 has any functional significance. However transfection of BM36.1 with each allele in turn will reveal whether they have any selective effect on the peptides that are presented by Class I molecules at the cell surface.

Claims

1. Double stranded nucleic acid containing the coding sequence of at least one of RING4, RING10, RING 11 and RING12 as set out in Figs 2 to 5 or a part thereof.

2. A protein having a sequence encoded by at least one of RING4, RING10, RING11 and RING12 as set out in Figs 2 to 5.

3. A nucleic acid encoding a protein or peptide having a sequence the same as or homologous to at least one of RING4, RING10, RING11 and RING12 or which his capable of interrupting the association of RING4 and/or RING11 subunits to form transporter peptides or which is capable of interrupting the association or RING10 and/or RING12 subunits with other components of the proteasome complex, or which is capable of preventing peptide transport by RING4/ RING11 transporter proteins or capable of preventing proteolysis by the proteasome complex by competitive binding to any one of RING4, RING11 and RING12 subunits, or which bears an epitope detected by antibodies capable of specifically recognising one of the RING4, RING10, RING11 and RING12 subunits, or a nucleic acid complementary thereto.

4. A protein or peptide having a sequence the same as or

homologous to at least one of the RING4, RING10, RING11 and RING12 expression products or which is capable of interrupting the association of RING4 and/or RING11 subunits to form transporter peptides, or which is capable of interrupting the association of RING10 and/or RING12 subunits with other components of the proteasome complex, or which is capable of preventing peptide transport by RING4/RING11 transporter proteins or capable of preventing proteolysis by the proteasome complex by competitive binding to any one of RING4, RING11 and RING12 subunits, or which bears an epitope detected by antibodies capable of specifically recognising one of the RING4, RING10, RING11 and RING12 subunits.

5. An antibody or fragment thereof capable of specifically recognising a protein according to claim 2 or a protein or peptide according to claim 4.

6. Nucleic acid encoding an antibody or fragment thereof according to claim 5.

7. A cell capable of secreting an antibody or fragment thereof according to claim 5.

8. A cloning or expression vector, virus genome, virus, transfected cell or transgenic animal containing exogenous nucleic acid according to any one of claims 1, 3 and 6.

9. A nucleic acid, protein, peptide, antibody, antibody-secreting, cell, vector, virus genome, virus, transfected cell or transgenic animal according to any one of claims 1 to 8 for use in a method of treatment of the human or animal body or in a method of diagnosis practised on the human or animal body.

10. Use of a nucleic acid protein, peptide, antibody, antibody-secreting, cell, vector, virus genome, virus, transfected cell or transgenic animal according to any one of claims 1 to 8 in the production of a medicament for use in a method of treatment of the human or animal body or in a method of diagnosis practised on the human or animal body.

11. A method of treating a diagnosis of a human or animal in need thereof comprising administering an effective, non-toxic amount of a nucleic acid, protein, peptide, antibody, antibody-secreting, cell, vector, virus genome, virus, transfected cell or transgenic animal according to any one of claims 1 to 8 to said human or animal.

12. A diagnostic test or assay which comprises the use of, or the detection of a nucleic acid protein, peptide, antibody, antibody-secreting, cell, vector, virus genome, virus, transfected cell or transgenic animal according to any

one of claims 1 to 8, in a test sample in vitro.

13. A compound or composition which diminishes or enhances antigen presentation in association with MHC Class I molecules by which is capable of interrupting the association of RING4 and/or RING11 subunits to form transporter peptides, or which is capable of interrupting the association of RING10 and/or RING12 subunits with other components of the proteasome complex, or which is capable of preventing peptide transport by RING4/RING11 transporter proteins or capable of preventing proteolysis by the proteasome complex by competitive binding to any one of RING4, RING11 and RING12 subunits.

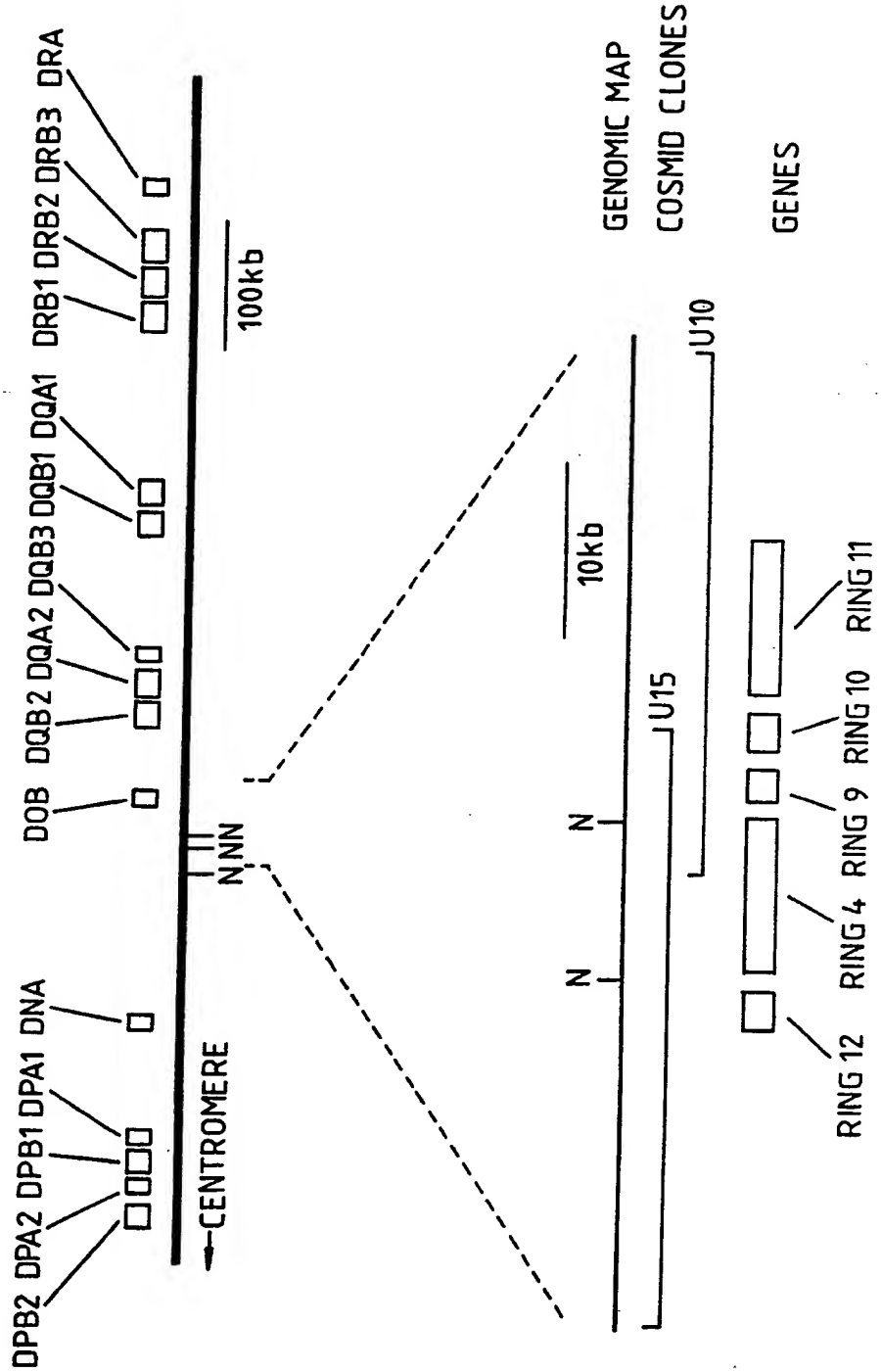
14. An assay for identifying a compound or composition according to claim 13 comprising simultaneously or sequentially in either order:

(i) contacting a cell capable of presenting antigenic peptides with a standard protein or peptide which is presented by the cell as a peptide: MHC Class I complex, and

(ii) contacting the cell with a compound or composition suspected to enhance or diminish antigen presentation

and thereafter assaying the peptide: MHC Class I complex presented by the cell.

Fig. 1.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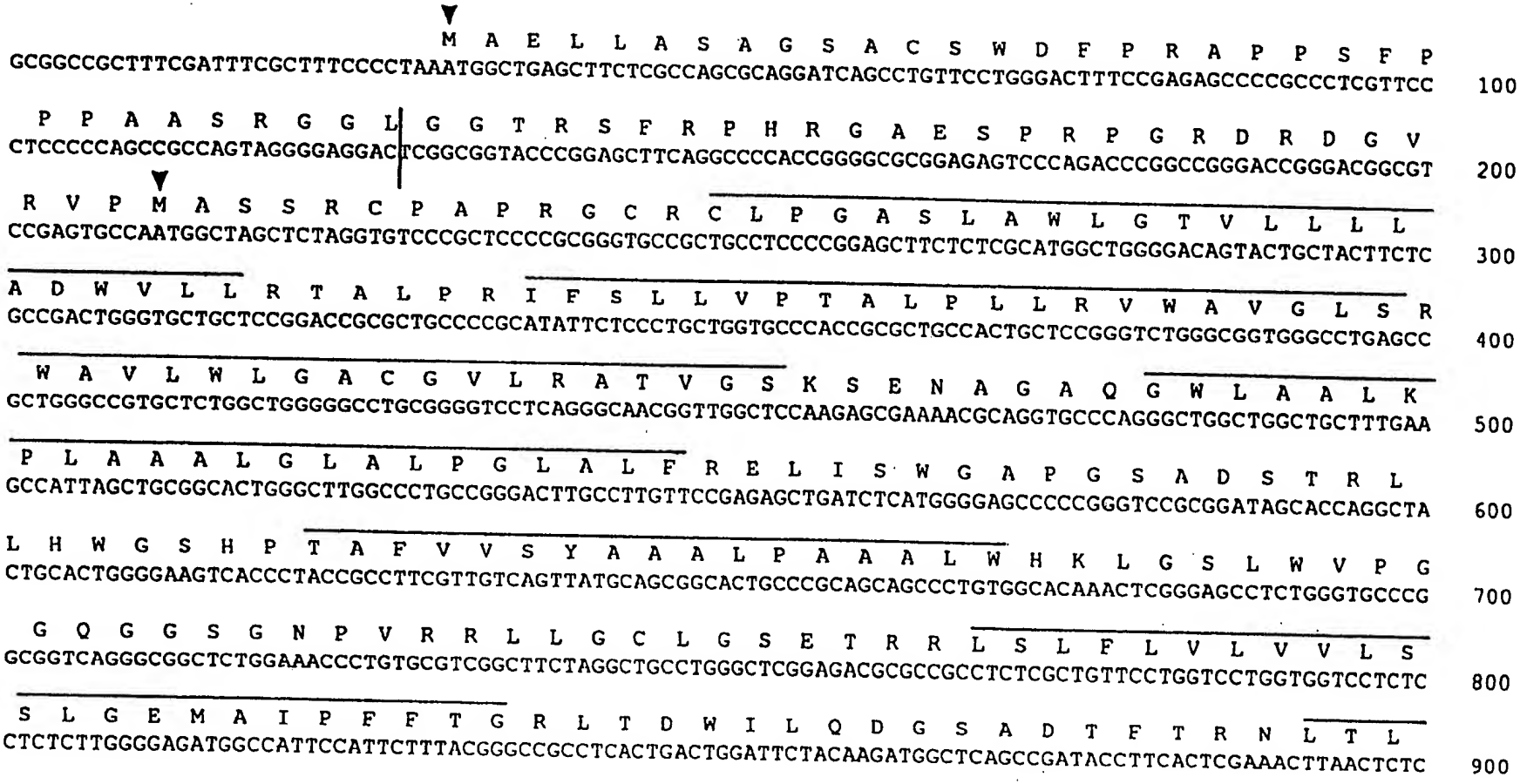
DRB3
DRA
CENTROMERE
N
N
N
100kb
10kb
GENOMIC MAP
COSMID CLONES
GENES
U15
U10
RING 12
RING 4
RING 9
RING 10
RING 11

Fig. 2.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P P A A S R G G L G G T R S F R P H R G A E S P R P G R D R D G V
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R V P M A S S R C P A P R G C R C L P G A S L A W L G T V L L L L
CCGAGTGCCAATGGCTAGCTCTAGGTGTCCCGCTCCCCGCGGGTGCCGCTGCCTCCCCGGAGCTTCTCTCGCATGGCTGGGGACAGTACTGCTACTTCTC 300
A D W V L L R T A L P R I F S L L V P T A L P L L R V W A V G L S R
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W A V L W L G A C G V L R A T V G S K S E N A G A Q G W L A A L K
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P L A A A L G L A L P G L A L F R E L I S W G A P G S A D S T R L
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Fig. 2(Cont 1).

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 S L S E N L S L F L W Y L V R G L C L L G I M L W G S V S L T M V
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T L I T L P L L F L L P K K V G K W Y Q L L E V Q V R E S L A K S S
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  Q V A I E A L S A M P T V R S F A N E E G E A Q K F R E K L Q E I
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 K T L N Q K E A V A Y A V N S W T T S I S G M L L K V G I L Y I G
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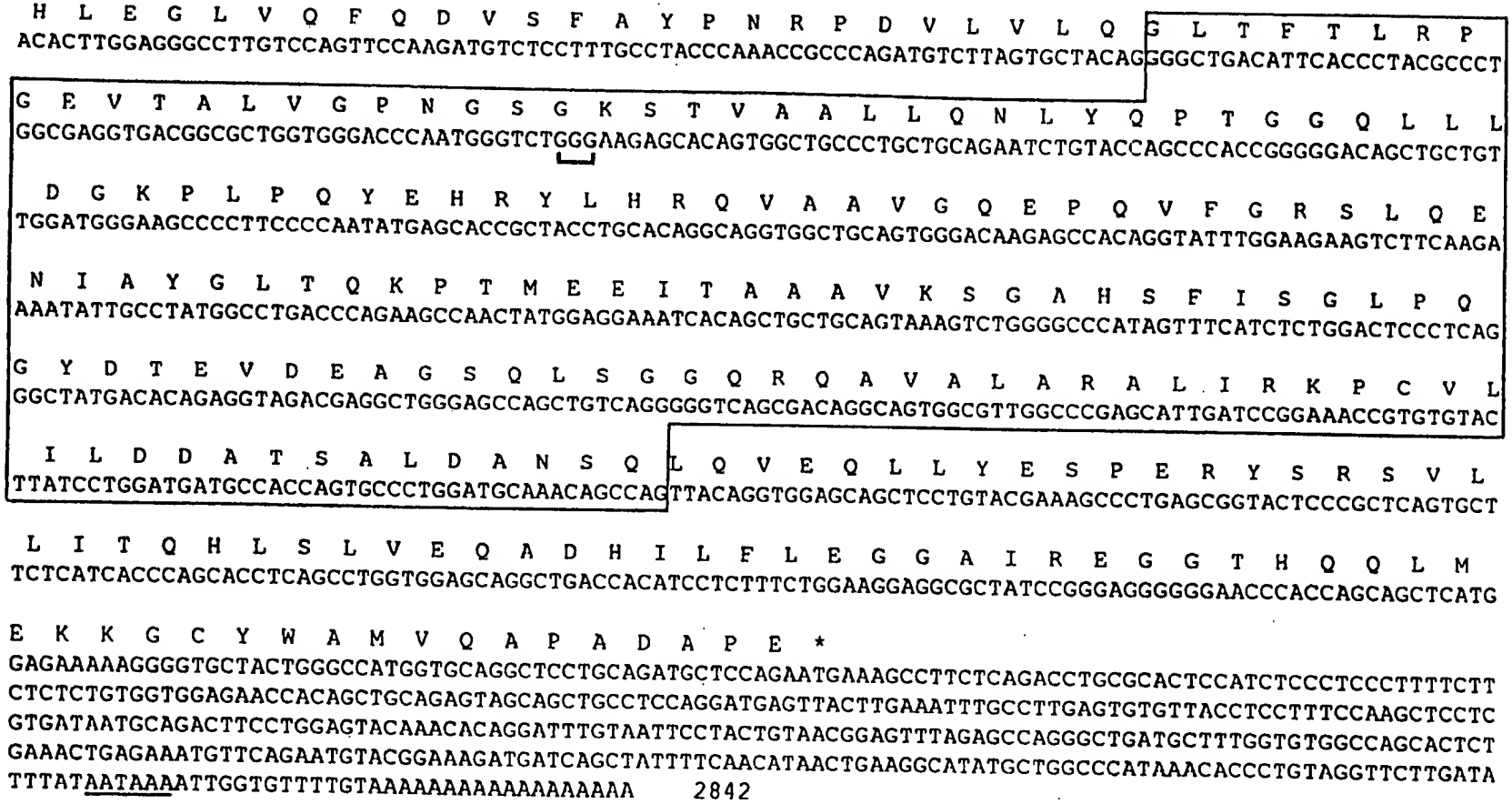
```

Fig. 2(Cont 2)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G E V T A L V G P N G S G K S T V A A L L Q N L Y Q P T G G Q L L L
GGCGAGGTGACGGCGCTGGTGGGACCCAATGGGTCTGGGAAGAGCACAGTGGCTGCCCTGCTGCAGAATCTGTACCAGCCCACCGGGGGACAGCTGCTGT 1900
D G K P L P Q Y E H R Y L H R Q V A A V G Q E P Q V F G R S L Q E
TGGATGGGAAGCCCCTTCCCCAATATGAGCACCGCTACCTGCACAGGCAGGTGGCTGCAGTGGGACAAGAGCCACAGGTATTTGGAAGAAGTCTTCAAGA 2000
N I A Y G L T Q K P T M E E I T A A A V K S G A H S F I S G L P Q
AAATATTGCCTATGGCCTGACCCAGAAGCCAACTATGGAGGAAATCACAGCTGCTGCAGTAAAGTCTGGGGCCCATAGTTTCATCTCTGGACTCCCTCAG 2100
G Y D T E V D E A G S Q L S G G Q R Q A V A L A R A L I R K P C V L
GGCTATGACACAGAGGTAGACGAGGCTGGGAGCCAGCTGTCAGGGGGTCAGCGACAGGCAGTGGCGTTGGCCCGAGCATTGATCCGGAAACCGTGTGTAC 2200
I L D D A T S A L D A N S Q L Q V E Q L L Y E S P E R Y S R S V L
TTATCCTGGATGATGCCACCAGTGCCCTGGATGCAAACAGCCAGTTACAGGTGGAGCAGCTCCTGTACGAAAGCCCTGAGCGGTACTCCCGCTCAGTGCT 2300
L I T Q H L S L V E Q A D H I L F L E G G A I R E G G T H Q Q L M
TCTCATCACCCAGCACCTCAGCCTGGTGGAGCAGGCTGACCACATCCTCTTTCTGGAAGGAGGCGCTATCCGGGAGGGGGGAACCCACCAGCAGCTCATG 2400
E K K G C Y W A M V Q A P A D A P E *
GAGAAAAAGGGGTGCTACTGGGCCATGGTGCAGGCTCCTGCAGATGCTCCAGAATGAAAGCCTTCTCAGACCTGCGCACTCCATCTCCCTCCCTTTTCTT 2500
CTCTCTGTGGTGGAGAACCACAGCTGCAGAGTAGCAGCTGCCTCCAGGATGAGTTACTTGAAATTTGCCTTGAGTGTGTTACCTCCTTTCCAAGCTCCTC 2600
GTGATAATGCAGACTTCCTGGAGTACAAACACAGGATTTGTAATTCCTACTGTAACGGAGTTTAGAGCCAGGGCTGATGCTTTGGTGTGGCCAGCACTCT 2700
GAAACTGAGAAATGTTCAGAATGTACGGAAAGATGATCAGCTATTTTCAACATAACTGAAGGCATATGCTGGCCCATAAACACCCTGTAGGTTCTTGATA 2800
TTTATAATAAAATTGGTGTTTTGTAAAAAAAAAAAAAAAAAA 2842

Fig. 3.

```
GGGCAGAAAGGGCACGCTCTTGTGGGTGACTACAGGTTAGGAGACCGTTGAACCTGGAGGGGCCCTAGGATGGACCCCGTGGAAAGATTCAGAGACTGCG  100

CCCTCTCCCTGGCGCCGCCTTCCCCTACACGCGGCGGGTATATTCTGTTGCAGTTGGCCCAGGACCTGTTTCCAAGACTCTGCCCCCTCGCACTTCCGTC  200

CCTCCTGGTTTTGTAAAGTGATGCTCATAGGAACCCCCACCCCGCGTGACACTACTCCCAGCTCCTGGCTGACTTCTAGTCTTCTGGTTGAAGCTGCGCC  300
                    M  L  I  G  T  P  T  P  R  D  T  T  P  S  S  W  L  T  S  S  L  L  V  E  A  A  P   27

TTTAGATGACACGACCCTACCCACCCCTGTTTCCAGCGGATGCCCGGGCCTGGAGCCCACAGAATTCTTCCAGTCCCTGGGTGGGGACGGAGAAAGGAAC  400
 L  D  D  T  T  L  P  T  P  V  S  S  G  C  P  G  L  E  P  T  E  F  F  Q  S  L  G  G  D  G  E  R  N   60

GTTCAGATTGAGATGGCCCATGGCACCACCACGCTCGCCTTCAAGTTCCAGCATGGAGTGATTGCAGCAGTGGATTCTCGGGCCTCAGCTGGGTCCTACA  500
V  Q  I  E  M  A  H  G  T  T  T  L  A  F  K  F  Q  H  G  V  I  A  A  V  D  S  R  A  S  A  G  S  Y  I   94

TTAGTGCCTTACGGGTGAACAAGGTGATTGAGATTAACCCTTACCTGCTTGGCACCATGTCTGGCTGTGCAGCAGACTGTCAGTACTGGGAGCGCCTGCT  600
  S  A  L  R  V  N  K  V  I  E  I  N  P  Y  L  L  G  T  M  S  G  C  A  A  D  C  Q  Y  W  E  R  L  L  127

GGCCAAGGAATGCAGGCTGTACTATCTGCGAAATGGAGAACGTATTTCAGTGTCGGCAGCCTCCAAGCTGCTGTCCAACATGATGTGCCAGTACCGGGGC  700
 A  K  E  C  R  L  Y  Y  L  R  N  G  E  R  I  S  V  S  A  A  S  K  L  L  S  N  M  M  C  Q  Y  R  G   160

ATGGGCCTCTCTATGGGCAGTATGATCTGTGGCTGGGATAAGAAGGGTCCTGGACTCTACTACGTGGATGAACATGGGACTCGGCTCTCAGGAAATATGT  800
M  G  L  S  M  G  S  M  I  C  G  W  D  K  K  G  P  G  L  Y  Y  V  D  E  H  G  T  R  L  S  G  N  M  F  194

TCTCCACGGGTAGTGGGAACACTTATGCCTACGGGGTCATGGACAGTGGCTATCGGCCTAATCTTAGCCCTGAAGAGGCCTATGACCTTGGCCGCAGGGC  900
  S  T  G  S  G  N  T  Y  A  Y  G  V  M  D  S  G  Y  R  P  N  L  S  P  E  E  A  Y  D  L  G  R  R  A  227

TATTGCTTATGCCACTCACAGAGACAGCTATTCTGGAGGCGTTGTCAATATGTACCACATGAAGGAAGATGGTTGGGTGAAAGTAGAAAGTACAGATGTC 1000
 I  A  Y  A  T  H  R  D  S  Y  S  G  G  V  V  N  M  Y  H  M  K  E  D  G  W  V  K  V  E  S  T  D  V   260

AGTGACCTGCTGCACCAGTACCGGGAAGCCAATCAATAATGGTGGTGGTGGCAGCTGGGCAGGTCTCCTCTGGGAGGTCTTGGCCGACTCAGGGACCTAA 1100
S  D  L  L  H  Q  Y  R  E  A  N  Q  *                                                                  272

GCCACGTTAAGTCCAAGGAGAAGAAGAGGCCTAGCCTGAGCCAAAGAGAGAGTAC...
```

Fig.4.

```
                                                1
      GCTGCGGTCTCCCCGCCGCGGCTGAGCCATG CGGCTCCCTGACCTG AGACCCTGGACCTCC CTGCTGCTGGTGGAC GCGGCTTTACTGTGG CTGCTTCAGGGCCCT
                                    *   M  R L P D L  R P W T S  L L L V D  A A L L W  L L Q G P
   79 CTGGGGACTTTGCTT CCTCAAGGGCTGCCA GGACTATGGCTGGAG GGGACCCTGCGGCTG GGAGGGCTGTGGGGG CTGCTAAAGCTAAGA GGGCTGCTGGGATTT
   27 L G T L L  P Q G L P  G L W L E  G T L R L  G G L W G  L L K L R  G L L G F
  184 GTGGGGACACTGCTG CTCCCGCTCTGTCTG GCCACCCCCCTGACT GTCTCCCTGAGAGCC CTGGTCGCGGGGGCC TCACGTGCTCCCCCA GCCAGAGTCGCTTCA
   62 V G T L L  L P L C L  A T P L T  V S L R A  L V A G A  S R A P P  A R V A S
  289 GCCCCTTGGAGCTGG CTGCTGGTGGGGTAC GGGGCTGCGGGGCTC AGCTGGTCACTGTGG GCTGTTCTGAGCCCT CCTGGAGCCCAGGAG AAGGAGCAGGACCAG
   97 A P W S W  L L V G Y  G A A G L  S W S L W  A V L S P  P G A Q E  K E Q D Q
  394 GTGAACAACAAAGTC TTGATGTGGAGGCTG CTGAAGCTCTCCAGG CCGGACCTGCCTCTC CTCGTTGCCGCCTTC TTCTTCCTTGTCCTT GCTGTCTTGGGTGAG
  132 V N N K V  L M W R L  L K L S R  P D L P L  L V A A F  F F L V L  A V L G E
  499 ACATTAATCCCTCAC TATTCTGGTCGTGTG ATTGACATCCTGGGA GGTGATTTTGACCCC CATGCCTTTGCCAGT GCCATCTTCTTCATG TGCCTCTTCTCCTTT
  167 T L I P H  Y S G R V  I D I L G  G D F D P  H A F A S  A I F F M  C L F S F
  604 GGCAGCTCACTGTCT GCAGGCTGCCGAGGA GGCTGCTTCACCTAC ACCATGTCTCGAATC AACTTGCGGATCCGG GAGCAGCTTTTCTCC TCCCTGCTGCGCCAG
  202 G S S L S  A G C R G  G C F T Y  T M S R I  N L R I R  E Q L F S  S L L R Q
  709 GACCTCGGTTTCTTC CAGGAGACTAAGACA GGGGAGCTGAACTCA CGGCTGAGCTCGGAT ACCACCCTGATGAGT AACTGGCTTCCTTTA AATGCCAATGTGCTC
  237 D L G F F  Q E T K T  G E L N S  R L S S D  T T L M S  N W L P L  N A N V L
  814 TTGCGAAGCCTGGTG AAAGTGGTGGGGCTG TATGGCTTCATGCTC AGCATATCGCCTCGA CTCACCCTCCTTTCT CTGCTGCACATGCCC TTCACAATAGCAGCG
  272 L R S L V  K V V G L  Y G F M L  S I S P R  L T L L S  L L H M P  F T I A A
  919 GAGAAGGTGTACAAC ACCCGCCATCAGGAA GTGCTTCGGGAGATC CAGGATGCAGTGGCC AGGGCGGGGCAGGTG GTGCGGGAAGCCGTT GGAGGGCTGCAGACC
  307 E K V Y N  T R H Q E  V L R E I  Q D A V A  R A G Q V  V R E A V  G G L Q T
 1024 GTTCGCAGTTTTGGG GCCGAGGAGCATGAA GTCTGTCGCTATAAA GAGGCCCTTGAACAA TGTCGGCAGCTGTAT TGGCGGAGAGACCTG GAACGCGCCTTGTAC
  342 V R S F G  A E E H E  V C R Y K  E A L E Q  C R Q L Y  W R R D L  E R A L Y
 1129 CTGCTCGTAAGGAGG GTGCTGCACTTGGGG GTGCAGATGCTGATG CTGAGCTGTGGGCTG CAGCAGATGCAGGAT GGGGAGCTCACCCAG GGCAGCCTGCTTTCC
  377 L L V R R  V L H L G  V Q M L M  L S C G L  Q Q M Q D  G E L T Q  G S L L S
```

Fig. 4 (Cont).

```
1234 TTTATGATCTACCAG GAGAGCGTGGGGAGC TATGTGCAGACCCTG GTATACATATATGGG GATATGCTCAGCAAC GTGGGAGCTGCAGAG AAGGTTTTCTCCTAC
 412 F  M  I  Y  Q   E  S  V  G  S   Y  V  Q  T  L   V  Y  I  Y  G   D  M  L  S  N   V  G  A  A  E   K  V  F  S  Y

1339 ATGGACCGACAGCCA AATCTGCCTTCACCT GGCACGCTTGCCCCC ACCACTCTGCAGGGG GTTGTGAAATTCCAA GACGTCTCCTTTGCA TATCCCAATCGCCCT
 447 M  D  R  Q  P   N  L  P  S  P   G  T  L  A  P   T  T  L  Q  G   V  V  K  F  Q   D  V  S  F  A   Y  P  N  R  P

1444 GACAGGCCTGTGCTC AAGGGGCTGACGTTT ACCCTACGTCCTGGT GAGGTGACGGCGCTG GTGGGACCCAATGGG TCTGGGAAGAGCACA GTGGCTGCCCTGCTG
 482 D  R  P  V  L   K  G  L  T  F   T  L  R  P  G   E  V  T  A  L   V  G  P  N  G   S  G  K  S  T   V  A  A  L  L

1549 CAGAATCTGTACCAG CCCACAGGGGGACAG GTGCTGCTGGATGAA AAGCCCATCTCACAG TATGAACACTGCTAC CTGCACAGCCAGGTG GTTTCAGTTGGGCAG
 517 Q  N  L  Y  Q   P  T  G  G  Q   V  L  L  D  E   K  P  I  S  Q   Y  E  H  C  Y   L  H  S  Q  V   V  S  V  G  Q

1654 GAGCCTGTGCTGTTC TCCGGTTCTGTGAGG AACAACATTGCTTAT GGGCTGCAGAGCTGC GAAGATGATAAGGTG ATGGCGGCTGCCCAG GCTGCCCACGCAGAT
 552 E  P  V  L  F   S  G  S  V  R   N  N  I  A  Y   G  L  Q  S  C   E  D  D  K  V   M  A  A  A  Q   A  A  H  A  D

1759 GACTTCATCCAGGAA ATGGAGCATGGAATA TACACAGATGTAGGG GAGAAGGGAAGCCAG CTGGCTGCGGGACAG AAACAACGTCTGGCC ATTGCCCGGGCCCTT
 587 D  F  I  Q  E   M  E  H  G  I   Y  T  D  V  G   E  K  G  S  Q   L  A  A  G  Q   K  Q  R  L  A   I  A  R  A  L

1864 GTACGAGACCCGCGG GTCCTCATCCTGGAT GAGGCTACTAGTGCC CTAGATGTGCAGTGC GAGCAGGCCCTGCAG GACTGGAATTCCCGT GGGGATCGCACAGTG
 622 V  R  D  P  R   V  L  I  L  D   E  A  T  S  A   L  D  V  Q  C   E  Q  A  L  Q   D  W  N  S  R   G  D  R  T  V

1969 CTGGTGATTGCTCAC AGGCTGCAGACAGTT CAGCGCGCCCACCAG ATCCTGGTGCTCCAG GAGGGCAAGCTGCAG AAGCTTGCCCAGCTC TAGGAGGGACAGGAC
 657 L  V  I  A  H   R  L  Q  T  V   Q  R  A  H  Q   I  L  V  L  Q   E  G  K  L  Q   K  L  A  Q  L   *

2074 CTCTATTCCCGCCTGGTGCAGCAGCGGCTGATGGACTGAGGCCCCAGGGATACTGGGCCCTCTTCTCAGGGGCGTCTCCAGGACCCAGAGCTGTTCCTGCTTTGAGTTTCC
2185 CTAGAGCTGTGCGGCCAGATAGCTGTTCCTGAGTTGCAGGCACGATGGAGATTTGGACACTGTGTGCTTTTGGTGGGGTAGAGAGGTGGGGTGGGGTGGGGTGGGGGCTGT
2296 CTGTGTCCAGGAAACTTAATTCCCTGGTGACTAGAGCTTTGCCTGGTGATGAGGAGTATTTTGTGGCATAATACATATATTTTAAAATATTTTCCTTCTTACATGAACTGT
2407 ATACATTCATATAGAAAATTTAGACAATATAAAAAAGTAC
```

Fig. 5.

```
                  M  L  R  A  G  A  P  T  G  D  L  P  R  A  G    15
CGGTGCCTTGCAGGGATGCTGCGGGCGGGAGCACCAACCGGGGACTTACCCCGGGCGGGA   60

E  V  H  T  G  T  T  I  M  A  V  E  F  D  G  G  V  V  M  G    35
GAAGTCCACACCGGGACCACCATCATGGCAGTGGAGTTTGACGGGGGCGTTGTGATGGGT  120

S  D  S  R  V  S  A  G  E  A  V  V  N  R  V  F  D  K  L  S    55
TCTGATTCCCGAGTGTCTGCAGGCGAGGCGGTGGTGAACCGAGTGTTTGACAAGCTGTCC  180
            R
P  L  H  E  H  I  Y  C  A  L  S  G  S  A  A  D  A  Q  A  V    75
CCGCTGCACGAGCACATCTACTGTGCACTCTCTGGTTCAGCTGCTGATGCCCAAGCCGTG  240
            cgc
A  D  M  A  A  Y  Q  L  E  L  H  G  I  E  L  E  E  P  P  L    95
GCCGACATGGCCGCCTACCAGCTGGAGCTCCATGGGATAGAACTGGAGGAACCTCCACTT  300

V  L  A  A  A  N  V  V  R  N  I  S  Y  K  Y  R  E  D  L  S   115
GTTTTGGCTGCTGCAAATGTGGTGAGAAATATCAGCTATAAATATCGAGAGGACTTGTCT  360

A  H  L  M  V  A  G  W  D  Q  R  E  G  G  Q  V  Y  G  T  L   135
GCACATCTCATGGTAGCTGGCTGGGACCAACGTGAAGGAGGTCAGGTATATGGAACCCTG  420

G  G  M  L  T  R  Q  P  F  A  I  G  G  S  G  S  T  F  I  Y   155
GGAGGAATGCTGACTCGACAGCCTTTTGCCATTGGTGGCTCCGGCAGCACCTTTATCTAT  480

G  Y  V  D  A  A  Y  K  P  G  M  S  P  E  E  C  R  R  F  T   175
GGTTATGTGGATGCAGCATATAAGCCAGGCATGTCTCCCGAGGAGTGCAGGCGCTTCACC  540

T  D  A  I  A  L  A  M  S  R  D  G  S  S  G  G  V  I  Y  L   195
ACAGACGCTATTGCTCTGGCCATGAGCCGGGATGGCTCAAGCGGGGGTGTCATCTACCTG  600

V  T  I  T  A  A  G  V  D  H  R  V  I  L  G  N  E  L  P  K   215
GTCACTATTACAGCTGCCGGTGTGGACCATCGAGTCATCTTGGGCAATGAACTGCCAAAA  660

F  Y  D  E  *                                                 219
TTCTATGATGAGTGAACCTTCCCCAGACTTCTCTTTCTTATTTTGTAATAAACTC       715
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Fig.6.

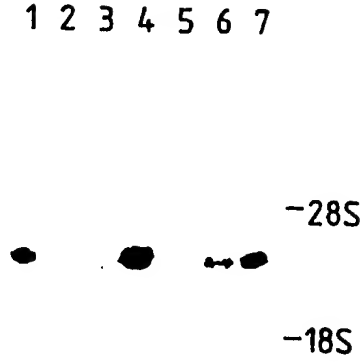


Fig.11.

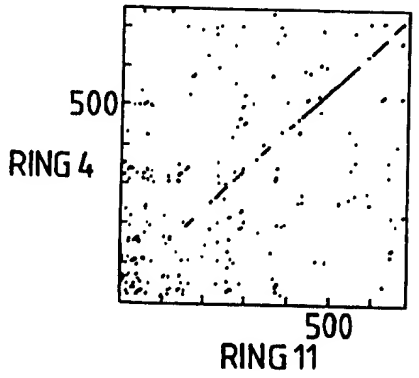


Fig.14.

	65					70					75					80				85					90				
RING10	M	A	H	G	T	T	T	L	A	F	K	F	Q	H	G	V	I	A	A	V	D	S	R	A	S	A	G	S	Y	I
HUM ε								-	-	-	-	-	R	X	-	-	-	v	-	a	-	-	-	-	t	-	-	G	-	F
RAT 6					-	-	I	-	-	-	-	-	-	E	-	-	-	l	-	X	-	-								
HUM δ							I	m	-	V	Q	-	d	G	-	-	v	l	G	a	-	-	-	T	t	T	-	-	-	-
RAT 5					-	-	I	m	-	V	Q	-	d	G	-	-	v	l	G	a	-	-								
RAT 7					-	-	I	m	-	V	E	-	d	G	-	-	v	v	G	S	-	-	D	v	-					
HUM β	-	v	T	-	-	s	V	-	G	V	-	-	e	G	-	-	v	i	-	a	-	M	L	G						
HUM C3	v	-	G	-	A	P	s	v	G	I	-	A	A	N	-	-	v	l	-	T	e	K	k	Q	K	S	I	L	-	D
Dm 35	v	K	L	-	-	A	-	v	G	L	-	N	K	D	Y	a	v	l	v	a	L	C	k	P	t	S	E	L	S	D
ScC1	v	E	N	-	-	-	s	i	G	I	-	C	n	D	-	-	v	F	-	-	e	K	L	i	t	S	K	L	L	v
TAα	v	K	K	-	s	-	A	-	G	M	-	-	A	N	-	-	l	l	i	S	-	K	k	v	R	S	R	L	I	E

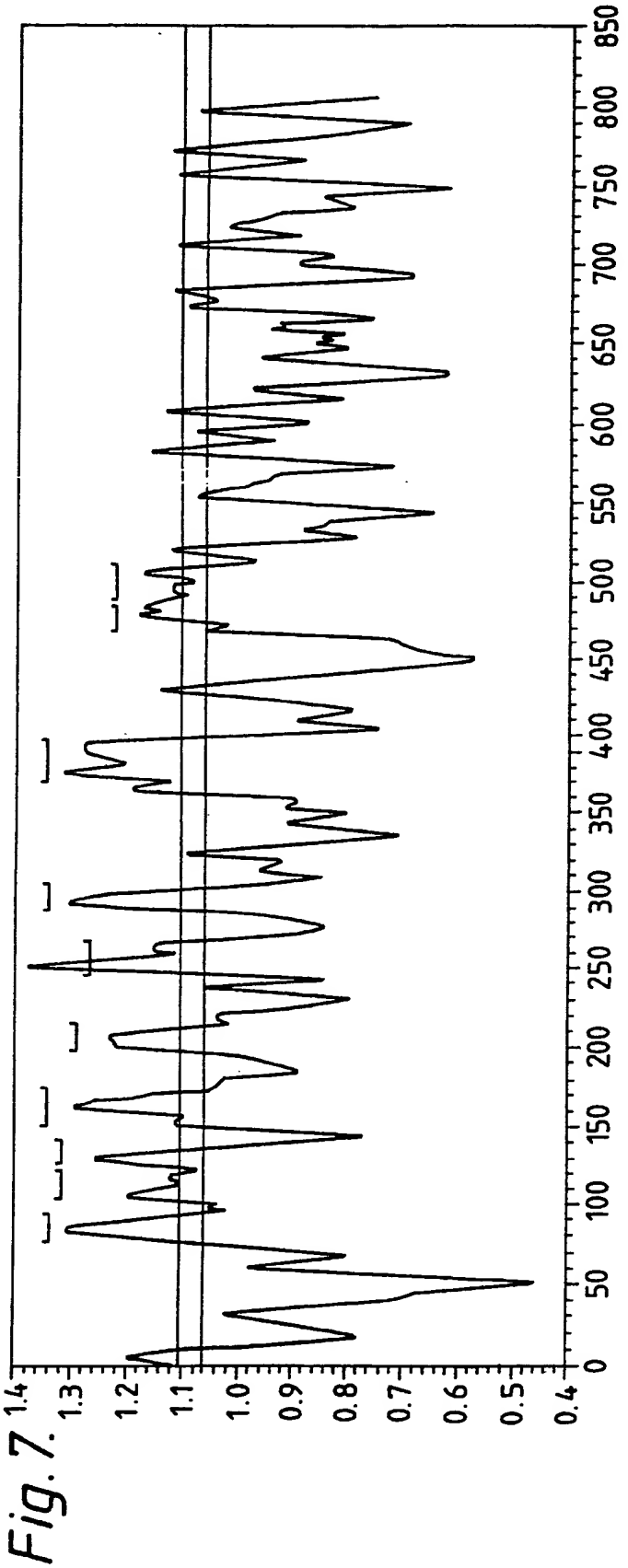


Fig. 7.

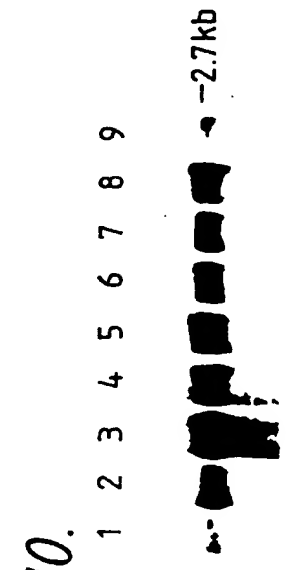


Fig.10.

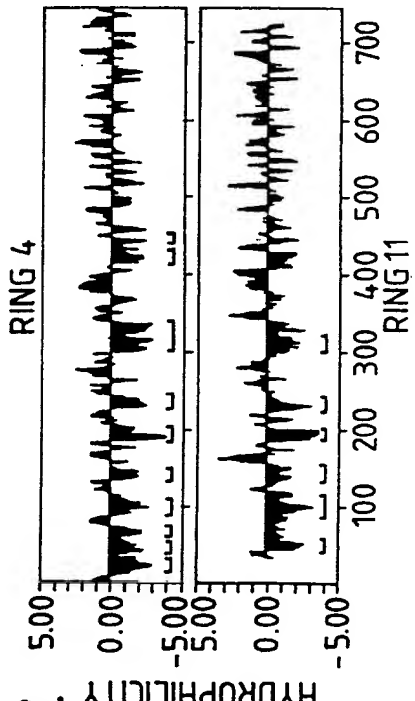


Fig. 9.

Fig.8.

```
RING4 GLTFTLRPGEVTALVGPNGSGKSTVAALLQN VAAVGQEPQVFGRSLQENIAYGLTQK DEAGSQLSGGQRQAVALARALIRKPCVLILDDATSALDANSQ

hmdr  --NLKVQS-QTV----NS-C----TVQ-M-R IGV-S---VL-ATTIA---R--RENV G-R-A------K-RI------V-N-KI-L--E------TE-E
      --SLEVKK-QTL----SS-C-----VQ--ER LGI-S---IL-DC-IA------DNSR GDK-T------K-RI-I----V-Q-HI-L--E------TE-E
ste6  NVSLNFSA-QF-FI--KS------LSN--LR ITV-E-RCTL-NDTLRK--LL-S-DS GTG-VT-----Q-R--I---F--DTPI-F--E-V----IVHR
      NMN-DMFC-QTLGII-ES-T----LVL--TK ISV-E-K-LL-NGTIRD-LT---QDE RIDTTL-----A-RLCI----L--SKI----EC-----SV-S
oppD  D-N----A--TLGI--ES-----QT-FA-MG ISMIF-D-MTSLNPYMRVGEQLMEVL KMYPHEF---M--R-MI-M--LCR-KL--A-EP-T---VTV-
oppF  -V-LR-YE--TLGV--ES-C----F-RAIIG IQMIF-D-LASLNPRMTIGEIIAEPL NRYPHEF---ECNRIGI-----LE-KLI-C---V----VSIQ
cyaB  NVSLRIA----VGV--RS------LTR-I-R LGV-L--STL-N--VRD---LTRPGA G-N-VG-------RIGI-----HR-R-----E------YE-E
hlyB  NINLSIKQ---IGI--RS------LTK-I-R -GV-L-DNVLLN--IID--SLANPGM G-Q-AG-------RI-I----VNN-KI-IF-E------YA-E
```

Fig.22(c).

```
        1864                    WB                ..
           GTACGAGACCCGCGGGTCCTCATCCTGGATGAGGCTACTAGTGCCCTAGATGTGCAGTGCGAGCAGGCCCTGCAGGACTGGAATTCCCGTGGGGATCGCACAGTG
R11A       V  R  D  P  R  V  L  I  L  D  E  A  T  S  A  L  D  V  Q  C  E  Q  A  L  Q  D  W  N  S  R  G  D  R  T  V
R11(36.1)                          ...E  A  T     C  P  R  C  A  V  R  A  G  P  A  G  L  E  F  P  W  G  S  H  S  A

           CTGGTGATTGCTCACAGGCTGCAGACAGTTCAGCGCGCCCACCAGATCCTGGTGCTCCAGGAGGGCAAGCTGCAGAAGCTTGCCCAGCTCTAGGAGGGACAGGAC
R11A       L  V  I  A  H  R  L  Q  T  V  Q  R  A  H  Q  I  L  V  L  Q  E  G  K  L  Q  K  L  A  Q  L  *
R11(36.1)     G  D  C  S  Q  A  A  D  S  S  A  R  P  P  D  P  G  A  P  G  G  Q  A  A  E  A  C  P  A  L  G  G  T  G  P

           CTCTATTCCCGCCTGGTGCAGCAGCGGCTGATGGACTGAGGCCCCAGGGATACTGGGCCCTCTTCTCAGGGGCGTCTCCAGGACCCAGAGCTGTTCCTGCTTTGA
R11(36.1)     L  F  P  P  G  A  A  A  A  D  G  L  R  P  Q  G  Y  W  A  L  F  S  G  A  S  P  G  P  R  A  V  P  A  L  S

           GTTTCCCTAGAGCTGTGCGGCCAGATAGCTGTTCCTGAGTT....
R11(36.1)     F  P  R  A  V  R  P  D  S  C  S  *
```

Fig.12.

```
          470                                                WA
RING11    FQDVSFAYPNRPDRP VLKGLTFTLRPGEVT ALVGPNGSGKSTVAA LLQNLYQPTGGQVLL DEKPISQYEHCYLHS QVVSVGQEPVLFSGS
RING4     -------------VL --Q------------ --------------- ------------L-- -G--LP-----R---R --AA------QV-SG-
HAM1      ----------Q-KVQ --Q------H--T-- --------------- ------------L-- -GQRLV--D-H---T --AA------L--GR-
mtp1      ----------H-NVQ --Q------Y--K-- --------------- -----------K--- -GE-LV--D-H---T --AA------L--GR-
Hmdr      -RN-H-S--S-KFVK I----NLKVQS-QTV ----NS-C----TVQ -M-R--D--E-M-SV -GQD-RTINVRF-RE IIGV-S------ATT

          560                                                                    WB
RING11    VRNNIAYGL*QSCED DKVMAAAQAAHADDF IQEMEHGIYTDVGEKG SQLAAGQKQRLAIAR ALVRDPRVLILDEAT SALD
RING4     LQE------T-KPTM EEIT---VKSG-HS- -SGLPQ-YD-E-D-A- ---SG--R-AV-L-- --I-K-C-----D-- ----
HAM1      F-E------NRTPTM EEIT-V-VESG-H-- -SGFPQ-YD-E---T- N--SG--R-AV-L-- --I-K-LL----D-- ----
mtp1      F-E------TRTPTM EEIT-V-MESG-H-- -SGFPQ-YD-E---T- N--SG--R-AV-L-- --I-K--L----D-- ----
Hmdr      IAE--R--R*ENVTM -EIEK-VKE-N-Y-- -MKLP-KFD-L---R- A--SG-----I---- ----N-KI-L----- ----
```

Fig.13.

```
          1969
RING11A     CTGGTGATTGCTCAC  AGGCTGCAGACAGTT  CAGCGCGCCCACCAG  ATCCTGGTGCTCCAG  GAGGGCAAGCTGCAG
RING11B     ---------------  ---------G-----  ---------------  ------------------------------
          657
RING11A   L  V  I  A  H   R  L  Q  T  V   Q  R  A  H  Q   I  L  V  L  Q   E  G  K  L  Q
RING11B   -  -  -  -  -   -  -  -  A  -   -  -  -  -  -   -  -  -  -  -   -  -  -  -  -

          2044
RING11A     AAGCTTGCCCAGCTC  TAGGAGGGACAGGAC  CTCTATTCCCGCCTG  GTGCAGCAGCGGCTG  ATGGACTGA
RING11B     ---------------C--------------  ---------------  --T------------  ---------
          682
RING11A   K  L  A  Q  L   *
RING11B   -  -  -  -  -   Q  E  G  Q  D   L  Y  S  R  L   V  Q  Q  R  L   M  D  *
```

Fig. 15.

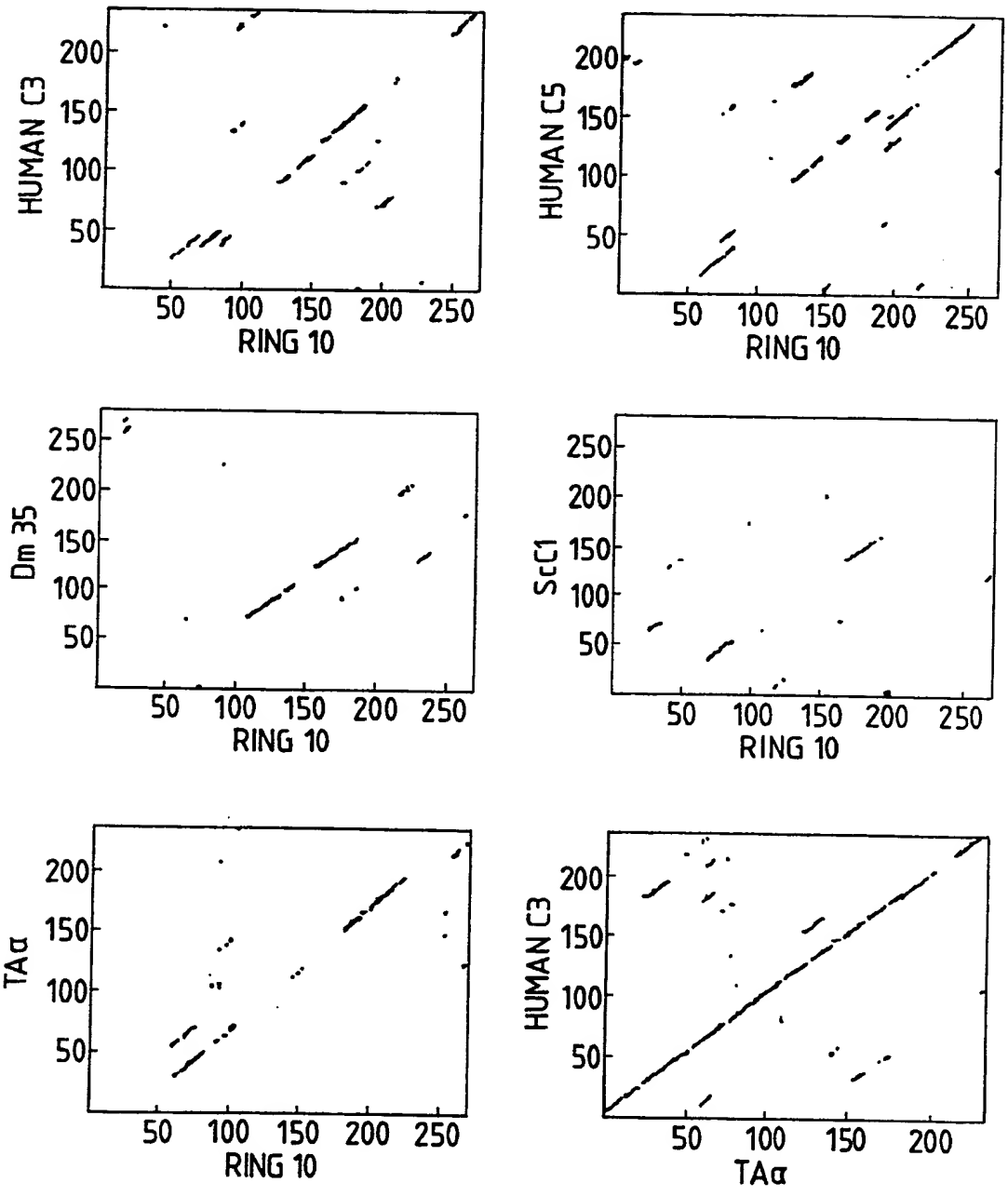

HUMAN C3
200
150
100
50
50 100 150 200 250
RING 10
HUMAN C5
200
150
100
50
50 100 150 200 250
RING 10
Dm 35
250
200
150
100
50
50 100 150 200 250
RING 10
ScC1
250
200
150
100
50
50 100 150 200 250
RING 10
TAα
200
150
100
50
50 100 150 200 250
RING 10
HUMAN C3
200
150
100
50
50 100 150 200
TAα

Fig.16.

	165					170					175					180					185				
RING10	M	G	S	M	I	C	G	W	D	K	K	G	P	G	L	Y	Y	V	D	E	H	G	T		
HUM C3	v	S	L	I	i	-	-	-	n	E	G	R	-	Y	-	f	Q	S	-	P	S	-	A		
HUM C5	v	Y	N	i	-	G	-	L	-	E	E	-	K	-	a	V	-	S	F	d	P	V	G		
Dm 35	v	-	L	I	v	a	-	y	-	-	E	r	-	-	H	i	-	-	Q	-	T	P	S	A	-
ScC1	v	S	L	i	F	G	-	V	-	-	N	-	-	A	H	-	-	M	I	e	P	S	-	s	-
TAα	v	S	L	i	F	a	-	I	-	Q	I	-	-	-	R	-	f	D	c	-	P	A	-	-	-

Fig.19.

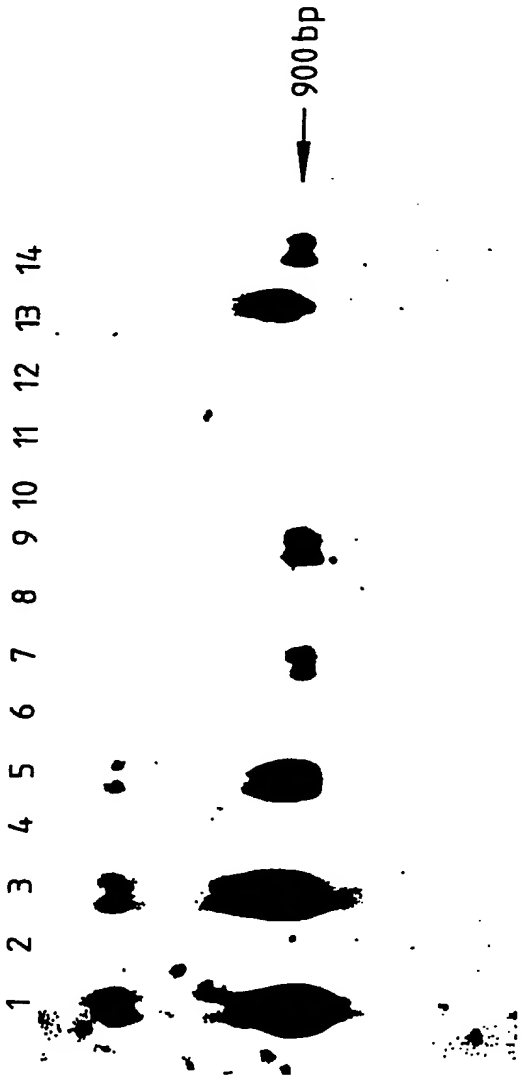


Fig.17.

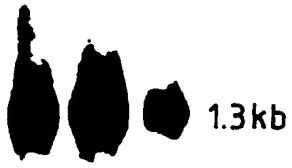
	162		*			
RING10	G	L	S	M	G	S
Subtilisin BPN'	-	T	-	-	A	-
Subtilisin S	-	T	-	-	A	t
furin/PACE	-	T	-	a	S	A
KEX2	-	T	-	a	A	A

	185								
	*								
RING10	H	G	T	R	L	S	G	N	M
Consensus	-	-	-	X	X	-	-	X	-
						t			l
						A			i
									v
									a

	206				
			*		
RING10	V	M	D	S	G
Subtilisin BPN	-	i	-	-	-
Subtilisin S	-	i	-	-	-
Furin/PACE	i	l	-	D	-
KEX2	i	v	-	D	-

Fig.18.

a) 1 2 3



b) - 2 48 72 96 hrs

Fig. 20.

```
                    aa19                                aa46
RING12              TGTTIMAVEFDGGVVMGSDSRVSAGEAV
RING10              A---TL-FK-QH--IAAV---A---SYI
RAT CHAIN 1           ---AG-VYKD-I
HUMAN A                 -AG-VYKD-I-L-A-TXATXVI
RAT CHAIN 2           ---AGLV-KD--IL-A-XEATN
HUMAN B             ---SVLG-K-E----IAA-MLG
RAT CHAIN 3           -QNPM-TGTSV--AKF
RAT CHAIN 4            SFSPYA-N--TLLVIXE
HUMAN G                FSPYV-N--TILAIAGEDF-IV-
RAT CHAIN 5           ------Q------L-A--
HUMAN D                 ----Q------L-A---TTT-SYI
RAT CHAIN 6           ---L-FK-QE--ILAX--
HUMAN E                  L-FK-RX--IVAA---AT--GYF
RAT CHAIN 7           -------------V----D--
```

Fig. 27.

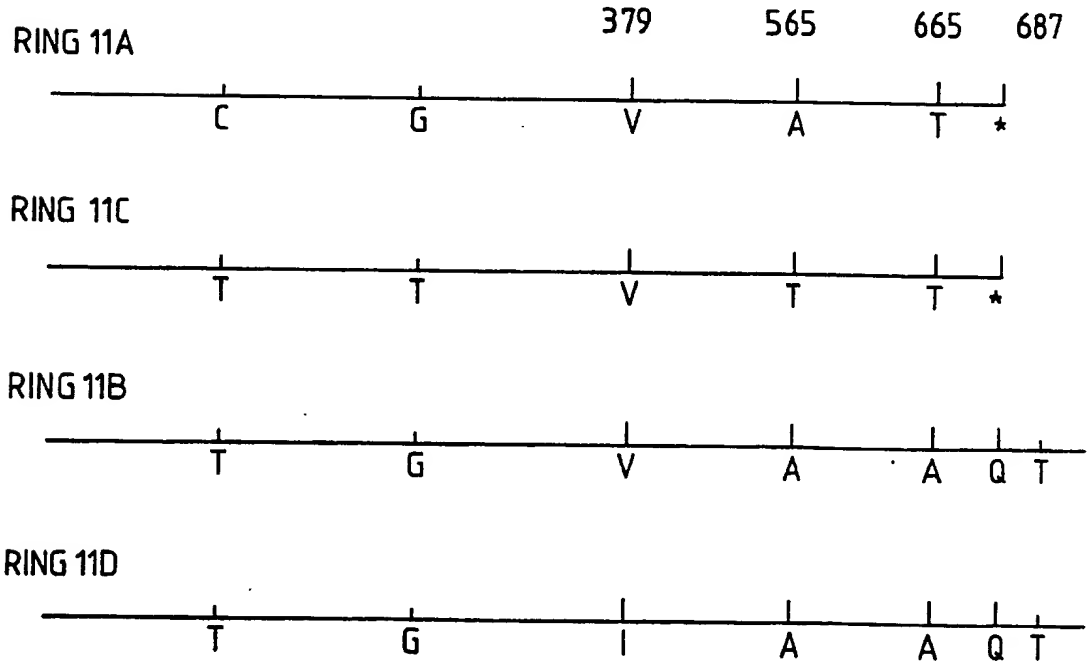


Fig. 21(a).

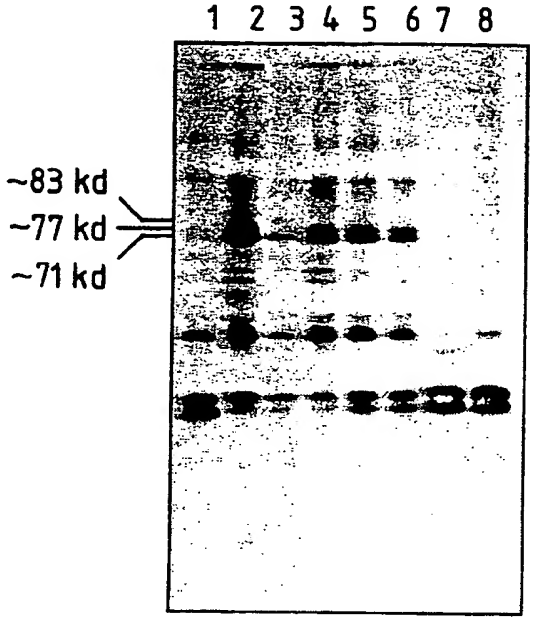


Fig. 21(b).

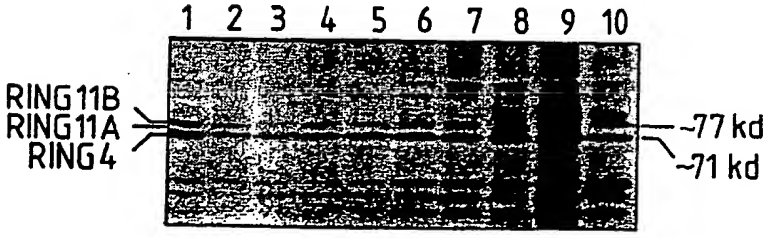


Fig. 22(a).

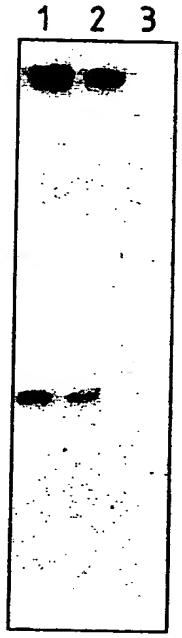


Fig. 22(b).

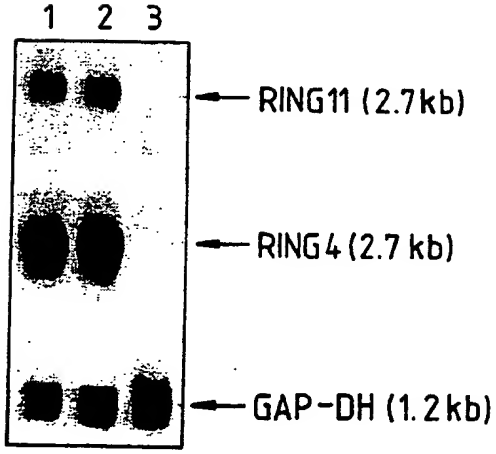


Fig. 23.

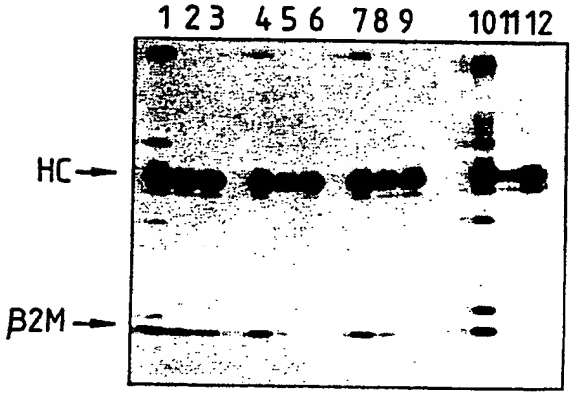


Fig. 24(a).

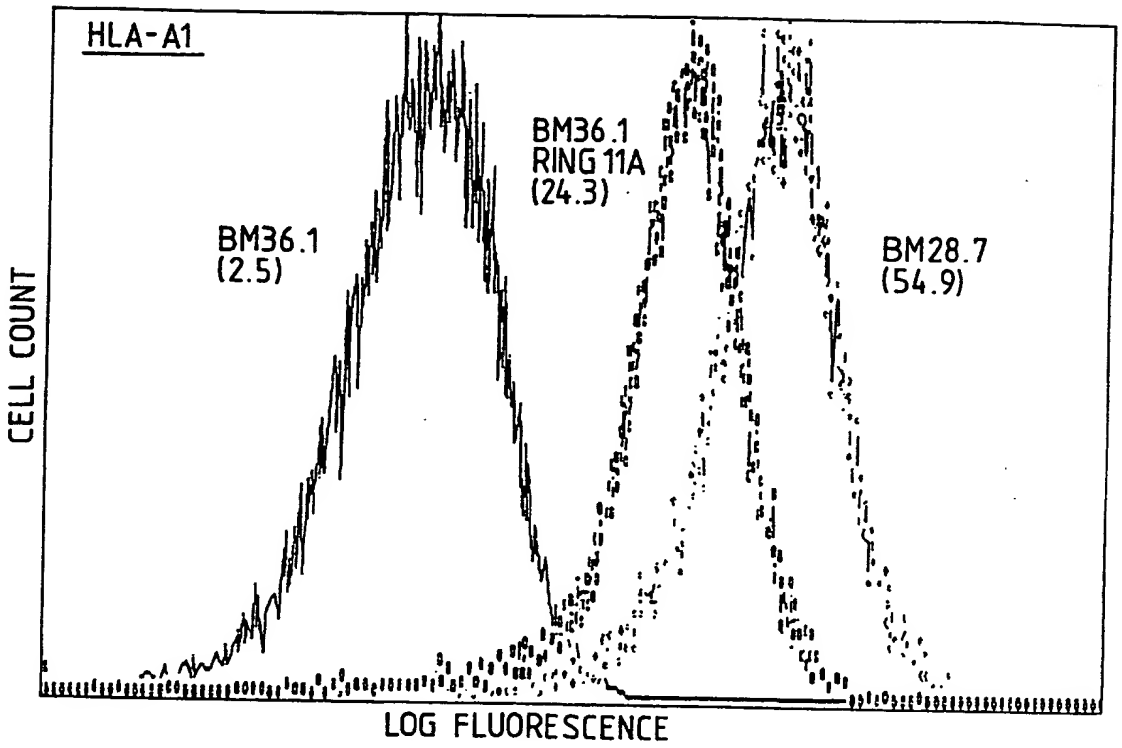
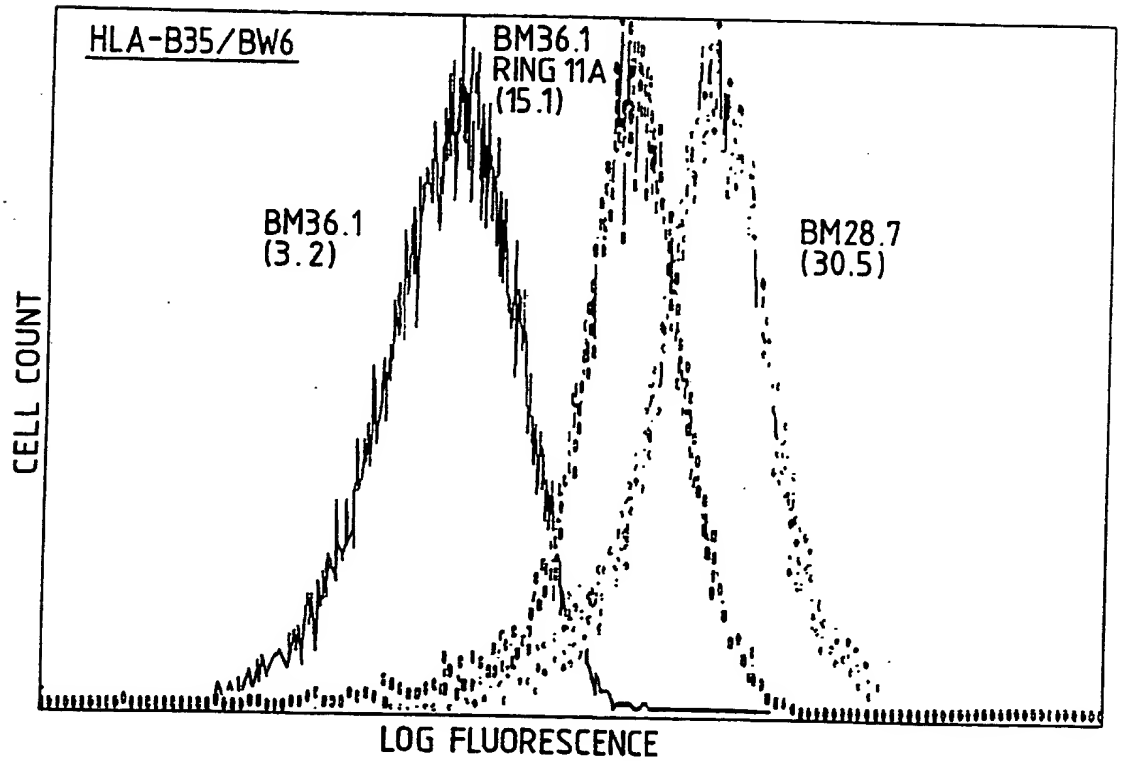


Fig. 24(b).



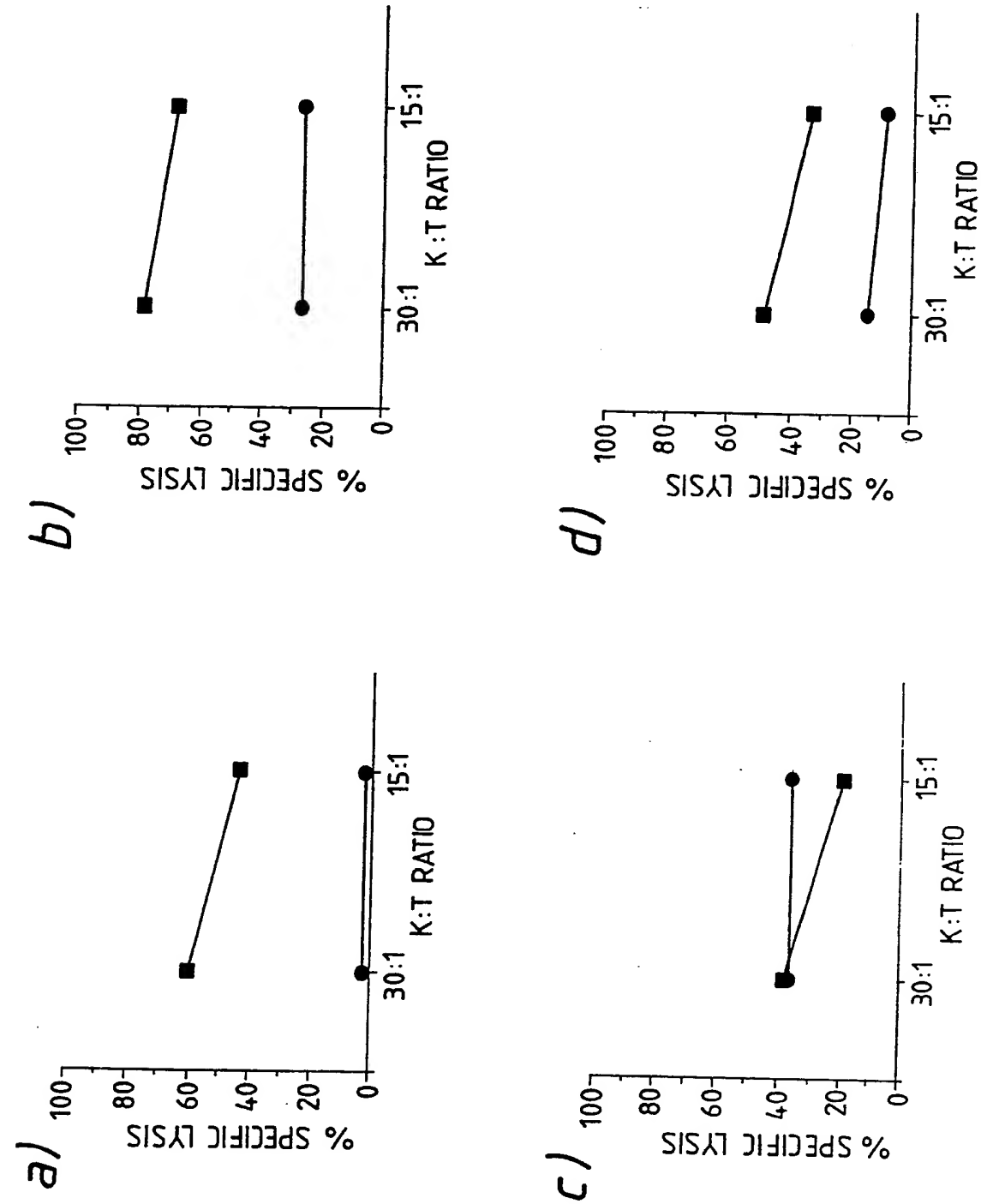
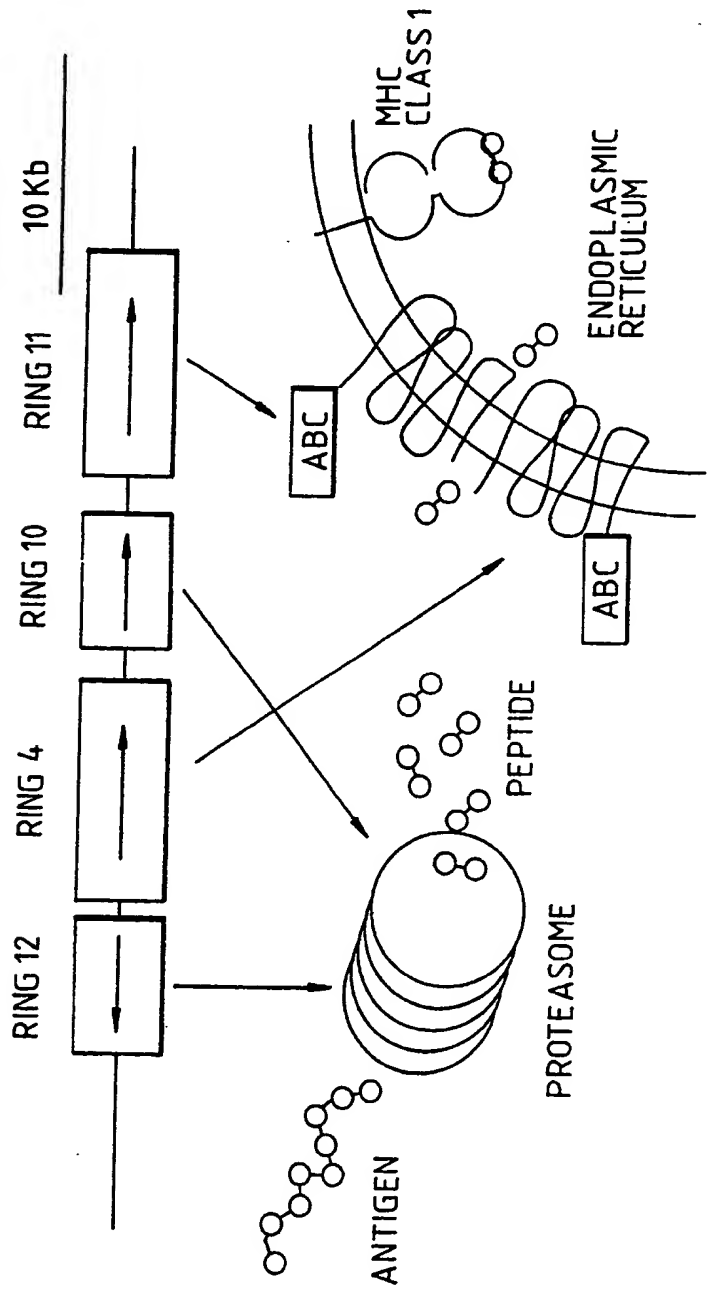


Fig.25.

Fig. 26.


10 Kb
RING 11
RING 10
RING 4
RING 12
ABC
ABC
MHC CLASS 1
ENDOPLASMIC RETICULUM
PEPTIDE
PROTEASOME
ANTIGEN

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/02278

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 13/00, C 12 N 15/12, G 01 N 33/53, C 12 Q 1/00 / A 61 K 39/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; C 12 N; G 01 N; C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
T	NATURE, vol. 348, No. 20/27, 1990, pages 674-675, Peter Parham: "Transporters of delight", see the whole article	1-10,12-14
A	NATURE, vol. 309, June 1984, John J. Monaco et al: "H-2-linked low-molecular weight polypeptide antigens assemble into an unusual marcromolecular complex ", see page 797 - page 799	1-10,12-14
A	NATURE, vol. 340, August 1989, Alain Townsend et al: "Association of class I major histocompatibility heavy and light chains induced by viral peptides ", see page 443 - page 448 see especially page 448 col. 1 line 20 - col. 2 line 45	1-10,12-14

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th March 1992	27 MAR 1992
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MISS I. TAZELAAR

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	NATURE, vol. 348, No. 20/27, December 1990, pages 741-744, John Trowsdale et al: "Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters", see especially fig. 3	1-10,12-14
P,A	NATURE, vol. 348, No. 20/27, December 1990, pages 744-747, Thomas Spies et al: "A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway", see especially fig. 4	1-10,12-14
P,X	NATURE, vol. 353, September 1991, Richard Glynne et al: "A proteasome-related gene between the two ABC transporter loci in the class II region of the human MHC ", see page 357 see especially fig. 2	1-10,12-14
P,A	NATURE, vol. 353, October 1991, Coleen K. Martinez et al: "Homology of proteasome subunits to a major histocompatibility complex-linked LMP gene ", see page 664 - page 667 see especially fig. 2	1-10,12-14
P,X	NATURE, vol. 353, October 1991, Adrian Kelly et al: "Second proteasome-related gene in the human MHC class II region ", see page 667 - page 668 see especially fig. 2	1-10,12-14

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [X] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [X] Claim numbers 11 it s/, because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body
c.f. PCT Rule 39.1.

2. [ ] Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional seach fees.